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Jerina, Helen

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# **Studies on Chronic Pain Mechanisms in the Central Nervous System**

**Helen Sarah Louise Jerina**

A thesis submitted for the degree of Doctor of Philosophy  
University of Bath  
Department for Health

May 2011

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## List of Abbreviations

µm	Micrometre
5HT	5-hydroxytryptamine
A	Anterior
ABC	Avidin biotinylated complex
ACC	Anterior cingulate cortex
ACR	American College of Rheumatology
ADP	Adenosine triphosphate
ALQ	Anterolateral quadrant
AMP	Adenosine monophosphate
ANOVA	Analysis of Variance
ASIC	Acid sensing ion channel
ATP	Adenosine triphosphate
Aβ	A beta
Aδ	A delta
Bk	Bradykinin
Ca <sup>2+</sup>	Calcium
CAR	Carrageenan
CCI	Chronic constriction nerve injury
cDNA	Complementary deoxyribonucleic acid
CFA	Complete Freund's adjuvant
CFS	Chronic fatigue syndrome
CGRP	Calcitonin gene-related peptide
CLBP	Chronic low back pain
CNS	Central nervous system
CRPS	Complex regional pain syndrome
CSF	Cerebrospinal fluid
CX3CL1	Chemokine (C-X3-C motif) ligand 1
CX3CR1	CX3C chemokine receptor 1
CXCL1	Chemokine (C-X-C motif) ligand 1
DAPI	4',6-diamidino-2-phenylindole
DCS	D-cycloserine
DLPFC	Dorsolateral prefrontal cortex
DNA	Deoxyribonucleic acid
DRG	Dorsal root ganglion
ELISA	Enzyme-linked immunosorbent assay
EPSP	Excitatory post synaptic potential
FITC	fluorescein isothiocyanate
FM	Fibromyalgia
g/mm <sup>2</sup>	Grams per square millimetre
GABA	Gamma-aminobutyric acid
GAP43	Growth associated protein 43
GFAP	Glial fibrillary acid protein
Hif1α	Hypoxia inducible factor one alpha
HPA	Hypothalamic-pituitary-adrenal

HSD	Honestly significant difference
i.p.	Intraperitoneal
IASP	International Association for the Study of Pain
ICC	Immunocytochemistry
ICV	Intracerebroventricular
IFA	Incomplete Freund's adjuvant
IgG	Immunoglobulin G
IL1	Interleukin 1
IL-1 AcP	Interleukin 1 accessory protein
IL-1R	Interleukin 1 receptor
IL-1Ra	Interleukin 1 receptor antagonist
IPSP	Inhibitory post synaptic potential
k.o.	Knock out
Kg	Kilogram
Kv	Potassium channel subunit
L	Left
LC	Locus Coeruleus
LPS	Lipopolysaccharide
LTP	Long term potentiation
m/s	Metres per second
MAP2	Microtubule associated protein 2
MAPK	Mitogen-activated protein kinase
MIA	Mechanically insensitive afferent
mPFC	Medial prefrontal cortex
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
MSA	Mechanically sensitive afferent
mV	Millivolts
MWM	Morris water maze
NA	Noradrenaline
NaV	Voltage gated sodium channel
Na <sub>v</sub> 1.8 -/-	Na <sub>v</sub> 1.8 knock out
NGF	Nerve growth factor
NMDA	N-Methyl-D-aspartic acid
NS	Nociceptive specific
NSAID	Non-steroidal anti-inflammatory drug
OCT	Optimum cutting temperature compound
P	Posterior
P2 <sub>x</sub>	Purinoreceptor
PAG	Peri-aqueductal grey
PAT	Passive avoidance test
PBMC	Peripheral blood monocyte
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline triton x100

PET	Positron emission tomography
PG	Prostaglandins
PHN	Post-hepatic neuralgia
PLC	Phospholipase C
PMA	Phorbol myristate acetate
PNS	Peripheral nervous system
PTSD	Post-traumatic stress disorder
R	Right
RAIC	Rostral area of the insular cortex
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RSD	Reflex sympathetic dystrophy
RT-PCR	Reverse transcription polymerase chain reaction
RVLM	Rostro-ventrolateral medulla
RVM	Rostro-ventral medulla
SE	Standard Error
SI	Primary somatosensory cortex
SII	Secondary somatosensory cortex
SNL	Spinal nerve ligation
SRT	Spinoreticular tract
STT	Spinothalamic tract
TA	Primer annealing temperature
TBS	Theta-burst stimulation
TENS	Transcutaneous electrical nerve stimulation
TM	Primer melting temperature
TNF $\alpha$	Tumour necrosis factor $\alpha$
TRP	Transient receptor potential cation channel
TRPV	Transient receptor potential Vanilloid cation channel
TTX	Tetrodotoxin
VGC	Voltage gated ion channel
VGCC	Voltage Gated Calcium Channel
VGKC	Voltage Gated Potassium Channel
VGSC	Voltage Gated Sodium Channel
VLO	Ventrolateral orbital region
VMN	Ventromedial nucleus of the hypothalamus
WDR	Wide dynamic range

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# Abstract

Chronic pain is one of the most significant medical and scientific challenges in western society today. As well as the emotional and physical effects on the individual it proves to be a significant financial burden to society. Studies have shown that chronic pain leads to central sensitisation that is partially regulated by release of proinflammatory molecules within the CNS. Most work has concentrated on the role of the spinal cord and little is known about changes in supraspinal regions. The CFA footpad model was used to investigate the expression of inflammatory mediators in the brain in persistent inflammatory pain.

Using RT-PCR, gene expression of inflammatory mediators was measured in various brain regions. Consistent with previous reports at 7 and 14 days post-injection IL1 $\beta$  expression was significantly elevated in the posterior of the brain ( $p<0.05$ ), TNF $\alpha$  showed differential expression with a significant increase in the posterior ipsilateral brain region at 72 hours ( $p<0.05$ ). The chemokine CXCL1 was significantly elevated at 6 hours post-injection ( $p<0.05$ ) suggesting a role for this chemokine in regulation of the acute pain response. Contrary to evidence from the spinal cord, CX3CL1 and its receptor CX3CR1 were down regulated in the brain at 6 and 24 hours post-injection. Differential expression of astrocyte activation was identified by GFAP immunohistochemistry.

Hypoxia has been implicated in neurodegeneration, a process thought to play a role in chronic pain. Here Hypoxia inducible factor (Hif1 $\alpha$ ) mRNA expression within the brain was not altered in a CFA model of peripheral inflammation. Interestingly, using Hypoxyprobe immunohistochemistry, a higher level of hypoxia was identified in non-injected controls than in CFA treated animals.

This is the first evidence of differential chemokine expression in the brain in persistent inflammatory pain and the first study to suggest a potential role for differential oxygenation within the brain in this condition.

# Chapter 1

## 1 Introduction: Pain Mechanisms

Pain is defined by the International Association for the Study of Pain (IASP) as “An unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage.” (Merskey and Bogduk, 1994). This definition goes some way to describe the complexity of pain, it is not merely a physical response to tissue damage, and it is a complex experience involving both sensory and emotional systems. These two components of pain are termed “sensory-discriminative” and “motivational-affective”, the former being the sensory and temporally descriptive response and the latter the emotional psychological side of the pain response, the element that makes pain “unpleasant and not just another sensation” (Melzack and Casey, 1968). These two components are both required for a sensation to be felt as pain and one can significantly impact on the strength of the other.

Although pain is useful, warning of potential damage or encouraging the protection of already damaged tissue to allow healing, it can also occur seemingly unnecessarily or persist long after damaged tissue has healed. The IASP definition of chronic pain is “Pain that persists beyond normal tissue healing time, which is assumed to be 3 months”. (Merskey and Bogduk, 1994). As well as conditions encompassed by this definition chronic pain can often result for no apparent reason.

Chronic pain affects 17-40% of the general population at some time and about 90% of cancer patients (Elliott, Smith *et al.* 1999; Blyth, March *et al.* 2001; Eriksen, Jensen *et al.* 2003; Jakobsson 2010). It therefore represents a significant medical and financial burden with many sufferers having to take a substantial amount of time off work. The existence of chronic pain and the significant differences between it and the acute pain response indicate the complexity of the somatosensory system. It is clear that nociception is not a



hardwired process, but a plastic one, wherein the nervous system can change and evolve in ways which are not always beneficial.

The classical understanding of the pain pathway involves a single three neuron chain, it is now known that the system is more complicated than this and involves at least two pathways at each level. These pathways are thought to control two different pain responses, the “first pain” which is usually a sharp pain occurring almost immediately after the stimulus and the “second pain” which is felt more slowly and tends to be a burning and less specifically localised sensation. As stated above, these two physical sensations can be described in terms of the “sensory-discriminative” and “affective-motivational” responses and will be discussed in further detail in the following chapters. It has been suggested that pain is not a physical perception at all but more an affectation or motivation to act, to protect already damaged tissue or to remove oneself from a potentially dangerous situation; a finger in a candle flame will start to hurt some time before there is any physical damage to the tissue.

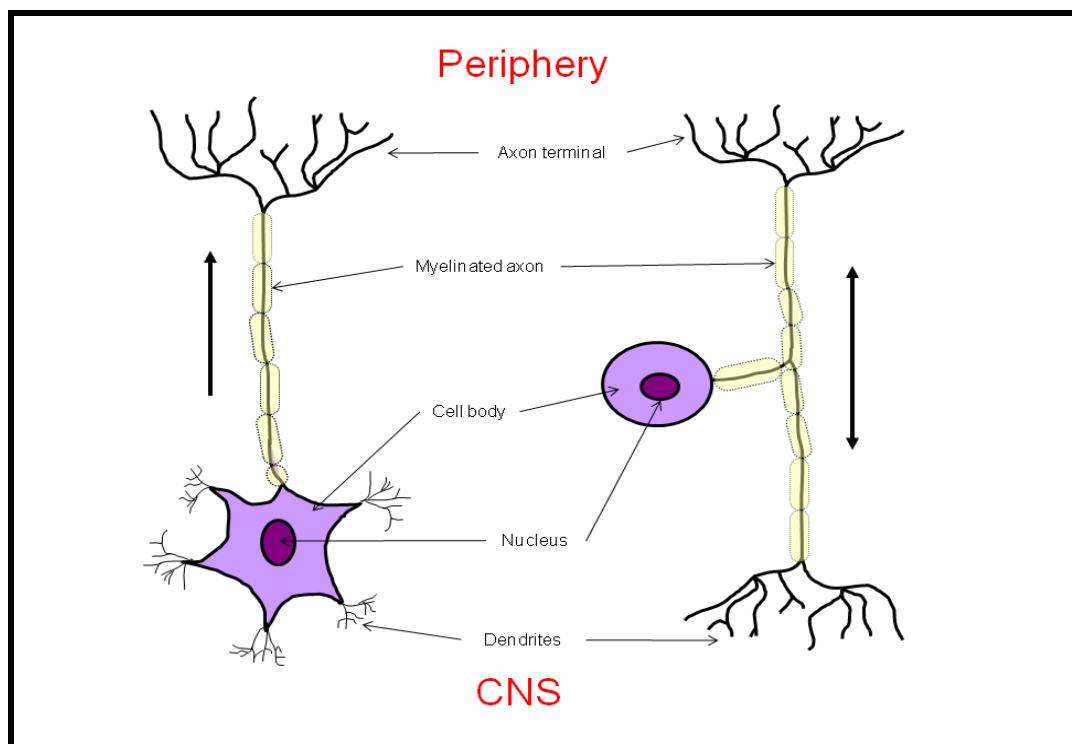
## **1.1 PNS Processing of Noxious Stimuli**

### **1.1.1 Nociceptive Nerve Fibres**

Nerves make up the “communication system” within the mammalian body, they are defined as either afferent or efferent; the former carrying information from the body to the CNS and the latter from the CNS to the body. Nerves are composed of a bundle of axons of varying size and structure which are specialised in carrying specific types of information; motor neurons are examples of efferent fibres and sensory neurons of afferent.

Primary afferent nerves which respond to painful or noxious stimuli are found throughout the body, particularly in the skin. These sensory afferents are known as nociceptors and activation of these receptors results in depolarisation of sensory neurons signalling damage to the CNS and resulting in a painful sensation. Nociceptive neurons differ from the accepted

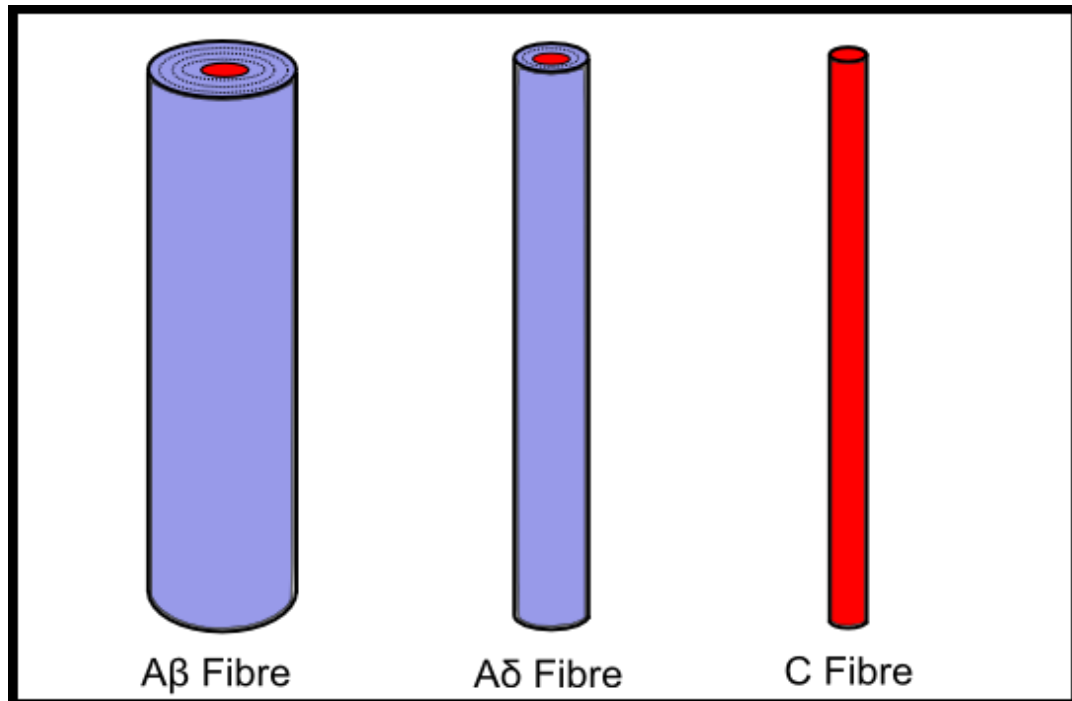
physiology of a “normal” neuron in that they can send and receive impulses in both directions. A standard neuron receives input at the dendrite and transmits information from its biochemically distinct axon, whereas, a nociceptive neuron can send and receive messages at either the peripheral end or the central terminus within the spinal cord. Nociceptive neurons are also unusual in that the cell body is contained outwith the axon in the dorsal root ganglion (DRG) of the spinal cord (or the trigeminal ganglion in the case of the face), proteins synthesised here are transported to both the peripheral and central terminals of the nociceptor, see figure 1. This bi-directionality means that, although the peripheral terminal is typically excited by the external stimulus and then sends a message to the central spinal terminal; both axonal and dendritic terminals can be regulated by endogenous and exogenous modulators.



**Figure 1 Diagram comparing a basic sensory neuron (right) with a “textbook” neuron such as a motor neuron (left).** The standard neuron is unidirectional, information is received by the dendrites and transmitted to the biochemically distinct axon terminus where proteins synthesised in the cell body are released. In contrast sensory neurons are bi-directional, receiving and transmitting information from either terminal. Proteins synthesised in the cell body, located in the DRG, can be released from either terminal.

Nociceptors are made up of three types of axon; C-fibres with small diameter (0.2-1.5µm) unmyelinated axons which conduct nerve impulses relatively

slowly (<2m/s), medium diameter (5-30  $\mu\text{m}$ ) myelinated A $\delta$  fibres conducting much faster at around 12 m/s, and finally large diameter ( $\sim$ 10 $\mu\text{m}$ ) A $\beta$  fibres which are also myelinated and conduct at speeds of up to 80m/s, see figure 2.



**Figure 2 The most common sensory fibres.** A $\beta$  fibres are large diameter (6-12 $\mu\text{m}$ ), myelinated axons carrying impulses at speeds of 35-75m/s, A $\delta$  fibres are medium diameter neurons (5-30 $\mu\text{m}$ ) carrying impulses at the slightly slower speed of 5-30m/s. These fibres are involved the faster first pain and mechanosensation. C-fibres are much smaller in diameter (0.2-1.5 $\mu\text{m}$ ) and transmit impulses at slower speeds (0.5-2m/s); they are associated with slow burning pain and itch.

The term “nociceptors” is something of a misnomer since the fibres described above do not only regulate painful sensations, some are pruriceptors mediating itch, others respond to innocuous temperatures and, recently, a subgroup of C-fibres has been identified that appears to mediate pleasant touch (Johansson *et al.*, 1988, Olausson *et al.*, 2010). In tissue injury and chronic pain some of these fibres regulating innocuous touch become hypersensitive to a variety of stimuli and contribute to the pain sensation, this has led to the term “silent nociceptor”.

Receptors exist for the transduction of many types of noxious stimuli; ion channels have been identified that open at specific temperatures or in response to mechanical or chemical stimulation. Nociceptors are polymodal;

they often express a variety of receptors and as such can respond to more than one stimulus. They also show heterogeneity in signalling mechanisms. Together this enables the encoding of multiple complex sensory stimuli into the incredible variety of sensations felt as pain.

Early studies used pinch to locate the receptive fields of nociceptors. However this method resulted in a subset of nociceptive neurons being ignored since not all nociceptors are mechanosensitive and, of those that are, many have very high thresholds. More recent studies using electrical stimulation to identify nociceptive fields in the cutaneous skin of monkeys and rats have demonstrated that approximately 50% of A $\delta$  fibres and 30% of C-fibres are mechanically insensitive afferents (MIAs) (Meyer *et al.*, 1991, Handwerker *et al.*, 1991) where MIAs were unresponsive to mechanical stimuli under 600g/mm<sup>2</sup>.

#### **1.1.1.1 C-fibres**

C-fibres, which primarily mediate the sensation of burning pain, are an example of the polymodality of nociceptors most of them responding to both heat and a variety of chemical stimuli. These fibres are generally excited by temperatures in the range of 39-41°C which corresponds to the human pain threshold to noxious heat (LaMotte and Campbell, 1978, Tillman *et al.*, 1995) and the activation temperature of the transient receptor potential vanilloid cation channel 1 (TRPV1 see 1.1.4.1) receptor (Caterina *et al.*, 1997) which is also activated by protons and the chemical capsaicin; TRPV1 and related receptors are discussed in more detail later in this chapter.

A subset of MIA C-fibres have been demonstrated to be involved in the regulation of itch, a process which shares many of the same pathways as nociception, and are excited by histamine, resulting in the characteristic inflammatory “wheal and flare” response (Schmelz *et al.*, 1997). There is significant evidence for an alternative subset of primary afferent pruriceptors since itch can be induced by mechanical and electrical stimulation (Ikoma *et al.*, 2005, Von Frey, 1922, Shelley and Arthur, 1957) as well as in response

to certain medications. Recently a seemingly non-inflammatory, itch mechanism has been identified where pruritogens, such as cowhage spicules from the *Mucuna pruriens* plant, directly activate C-fibres leading to an itch response with no flare (Schmelz, 2010, Johanek *et al.*, 2008, Namer *et al.*, 2008).

Another subset of C-fibres are activated by low-threshold mechanical stimulation which activate the insular, but not the somatosensory, cortex and are thought to be involved in mediating the sensation of pleasant touch (Johansson *et al.*, 1988, Olausson *et al.*, 2010). The discovery of these fibres is interesting as for many years mechanical stimulation was mainly considered to be mediated by A $\beta$ -fibres and A $\delta$ -fibres. The slow response of C-fibres and the activation of the insular cortex and limbic system by these mechanoreceptors indicates that they may have an affective/emotional role in the regulation of innocuous touch. It is possible that pleasant touch may have two functional responses as described for pain, via a sensory-discriminative and affective-motivational pathway (McGlone and Reilly, 2010).

Although C-fibres appear to have a limited role in mechanical pain a subset of generally MIA C-fibres appear to develop a response to mechanical stimuli following persistent mechanical stimulation suggesting they may have a role in regulating pain in response to tonic pressure. As mentioned previously C-fibres which only respond to very high pressures are also considered to be mechanically insensitive; such fibres may have a role in pain where their threshold of activation may be decreased by endogenous modulators (Basbaum *et al.*, 2009).

#### **1.1.1.2 A-fibres**

A-fibres are generally considered to mediate the pricking and sharp pain felt immediately after peripheral insult. They provide a faster more precise signal than C-fibres and are also thought to contribute to aching pain (Slugg *et al.*, 2000). The A-fibres are divided into two groups; type-I and type-II. Briefly, type-I fibres are sensitive to mechanical and chemical stimuli as well as

noxious heat in the higher range of noxious temperature ( $>53^{\circ}\text{C}$ ) as regulated by TRPV2 (Caterina *et al.*, 1999). Two types of fibre make up the type-I group,  $\text{A}\delta$  and  $\text{A}\beta$  fibres making this group the fastest conducting with speeds of 25-55m/s (McGlone and Reilly, 2010).

Type II fibres lay undiscovered for some time due to the fact that most of them are MIAs. Using an electrical stimulus as opposed to a pinch test to locate afferents demonstrated that there are roughly equal numbers of Type I and II fibres in non-glabrous primate skin. Type I fibres have similar properties to C-fibres in that they have a similar response to heat and may express the TRPV1 receptor. Since they are only thinly myelinated, type II fibres have relatively low conduction velocities, around 15m/s, compared to their type I counterparts (Treede *et al.*, 1995).

### **1.1.2 Sensory Neurotransmitters**

The predominant excitatory neurotransmitter used by all primary afferent fibres including nociceptive neurons is glutamate, although other transmitters also have a role in transmission and modulating excitability. As well as delivering information from the periphery to the CNS, primary afferent neurons release neurotransmitters within their peripheral locality where they have a significant role in neurogenic inflammation via the release of substance P and calcitonin gene related peptide (CGRP). A significant number of small fibre neurons, thought to be C-fibres, release such neuropeptides, not only in the periphery but also centrally, within the dorsal horn on stimulation where their action on postsynaptic receptors plays a key role in setting the magnitude of response of second order neurons (Basbaum *et al.*, 2009).

### **1.1.3 Ion Channels**

#### **1.1.3.1 Voltage Gated Ion Channels**

Following detection of a sensation in the periphery the signal must be propagated along the axon to the spinal cord, this signal takes the form of an action potential which is mediated by voltage gated ion channels (VGCs). Such channels regulate nerve impulses throughout the PNS and CNS and although some are ubiquitous, others are specific to a certain kind of neuron of sensory modality. VGCs have been identified that are specific to and play significant roles in nociceptive neurons, providing a greater understanding of the mechanisms of pain regulation and potential targets for medication.

#### **1.1.3.2 Sodium Channels**

Nine different types of voltage sensitive sodium channel have been identified in mammals and sensory neurons express six of these at relatively high levels (Ekberg and Adams, 2006) Voltage gated sodium channels consist of a single  $\alpha$  subunit made up of four membrane spanning domains which form the sodium specific pore; various  $\beta$  subunits and accessory proteins form the remainder of the channel and can contribute to its properties. It is by these  $\alpha$  subunits that the nine classes of sodium channel are classified as  $\text{Na}_v1.1$  –  $\text{Na}_v1.9$  (Goldin *et al.*, 2000). Sodium channels are also classified according to their susceptibility to blocking by the neurotoxin tetrodotoxin (TTX) with  $\text{Na}_v1.5$ ,  $\text{Na}_v1.8$  and  $\text{Na}_v1.9$  being TTX resistant while the remainder are irreversibly blocked following exposure.

Using electrophysiology sensory neurons have been shown to express both TTX-sensitive and TTX-resistant  $\text{Na}_v$  channels and the former have been demonstrated to be a prerequisite for action potential firing in most, if not all, of these neurons (Ritter and Mendell, 1992).

The action potential of sensory neurons differs from the “standard” action potential shape by having an extended shoulder in the depolarising phase.

This alters the electrophysiological properties of the neuron, such that, the falling phase is extended and the neuron takes longer to repolarise, resulting in longer lasting irregular action potentials (Koltzenburg *et al.*, 1997). Voltage gated sodium channels (VGSC) have been demonstrated to be significant in the regulation of this unusual action potential and specific channel isoforms are required for each phase of the response.

Following immunohistochemical and electrophysiological analysis, the specific Na<sub>v</sub> channel subtypes necessary for nociceptive function have been identified. As mentioned above, TTX-sensitive fibres are essential for action potential generation in all sensory neurons in nociceptive fibres; the channels demonstrated to have a significant role are Na<sub>v</sub>1.6 in myelinated axons and Na<sub>v</sub> 1.7 in unmyelinated nerves. The TTX-resistant channels, although non-essential for action potential generation, are vital in defining the specific electrophysiological properties of the nociceptive neuron (Caldwell *et al.*, 2000, Djouhri *et al.*, 2003b). Of the TTX-resistant channels Na<sub>v</sub>1.8 and Na<sub>v</sub>1.9 have been demonstrated to be expressed in most, if not all nociceptors and Na<sub>v</sub>1.9 is specific to nociceptive neurons (Djouhri *et al.*, 2003a, Fang *et al.*, 2002).

The absence of specific antagonists for a large number of VGSCs and the ubiquitous nature of others has made their study difficult. However, gene deletion studies in mice have enabled the identification of three genes encoding for specific sodium channels which are expressed in sensory neurons. SCN10A and SCN11A are nociceptor specific genes encoding the Na<sub>v</sub>1.9 and Na<sub>v</sub>1.8 channel  $\alpha$  subunits respectively. The third gene to be identified in nociceptors is SCN9A which encodes the Na<sub>v</sub>1.7 channel  $\alpha$  subunit (Foulkes and Wood, 2008).

The specific kinetic properties of Na<sub>v</sub>1.8 and Na<sub>v</sub>1.9 are the driving force behind the distinctive electrophysiological fingerprint of the nociceptive neuron. Na<sub>v</sub>1.9, unusually for a VGSC, opens at negative membrane potentials ( $\sim -80\text{mV}$ ) and remains open for a considerable time as the membrane potential increases then rapidly closes after the action potential



peak. This provides a small but persistent current at resting membrane potential enabling faster depolarisation of the neuron on stimulation (Herzog *et al.*, 2001).

Na<sub>v</sub>1.9 alone cannot explain the classical TTX-resistant current seen in sensory neurons as this is activated at a positive voltage and inactivates relatively slowly. This points to Na<sub>v</sub>1.8 as the other TTX-resistant VGSC expressed in nociceptors and indeed this channel has been demonstrated to be key in the nociceptive response following the generation of mice lacking Na<sub>v</sub>1.8 gene (Na<sub>v</sub>1.8 <sup>-/-</sup>) (Akopian *et al.*, 1999). The sensory neurons from the DRG of these mice expressed only rapidly activating and inactivating TTX-resistant currents and no slow VGSC currents were seen in DRG neurons from these animals and although these mice displayed normal physical characteristics they had decreased behavioural responses to both mechanical and thermal noxious stimuli and a delayed response to inflammatory hyperalgesia induced by carrageenan injection.

Sodium channels play a key role in setting neuronal excitability and their expression in peripheral nerves is often altered in pain states. Following experimental neuroma generation only 0.4% saphenous nerves from Na<sub>v</sub>1.8 k.o. mice exhibited ectopic firing compared to 19% of wild type counterparts (Roza *et al.*, 2003). The Na<sub>v</sub>1.8 k.o. mice also showed reduced mechanosensitivity in the early stages of the experiment (up to 10 days) which returned to normal by day 22 clearly implicating the Na<sub>v</sub>1.8 channel in the development and propagation of neuropathic pain. This demonstrates the plasticity of distribution of these channels and how trafficking of receptor and channel proteins may contribute to pain states.

In neuropathic injury and chronic pain conditions abnormal trafficking of channel proteins may lead to accumulation of sodium channels not only in the affected nerve but also in healthy neighbouring neurons. This increase in excitatory sodium channels will lead to a decreased activation threshold of the nerves local to the damaged resulting in an increase in the likelihood of ectopic firing, this signal propagation may contribute to central sensitisation.

Sodium channels have also been implicated in human pain conditions. Perhaps the best understood of these is familial erythromelalgia, a disorder affecting the peripheral neurovasculature leading to inflammation and pain, which is caused by a gain of function mutation in the SCN9A gene which codes for the  $\alpha$ -subunit of Na<sub>v</sub>1.7 (Cummins *et al.*, 2004). Conversely a loss of function mutation in humans results in an apparently normal phenotype except that these individuals cannot feel pain, a condition that can lead to serious or even life threatening injury (Cox *et al.*, 2006). Interestingly, global deletion of the SCN9A gene in mice causes them to die shortly after birth, apparently due to problems feeding, probably caused by loss of function of the sensory neurons (Nassar *et al.*, 2004).

#### **1.1.3.3 Calcium Channels**

Voltage gated calcium channels (VGCCs) act as translators of a message from one cell to the next, they open following depolarisation of the membrane allowing a massive intracellular calcium influx. This influx results in the contraction of muscle or release of hormones, neurotransmitters or other ligands from neurons. VGCCs contribute to increased nociceptive input from the periphery in both neuropathic and inflammatory pain, their role in neurotransmitter release at the first synapse means they are vital in the transmission of pain information from the periphery to the CNS. As well as signalling information from the periphery to the CNS and local motor circuits calcium release is also involved in descending regulation from the brain, a process that will be discussed in greater detail in Chapter 2.

There are a large variety of VGCCs, many of which have a significant role in neuronal function. They are divided into five subtypes; L, N, P, R and T, with N, P and R groups implicated in neurotransmission (Tsien *et al.*, 1995). Of these N and P type channels are implicated in painful conditions, the former in severe persistent pain and the latter in migraine (Julius and Basbaum, 2001). Subtype T VGCCs are also involved in neuronal function where they play a role in the electrophysical properties of neurons. The L-type VGCCs

regulate muscle contraction as well as neuronal plasticity and gene regulation (Basbaum *et al.*, 2009).

The individual subtypes of VGCC are differentially distributed throughout the body and N-type VGCC have been demonstrated to be critical for the majority of fast neurotransmission within sensory neurons (Gruner and Silva, 1994). N-type VGCC are largely present in the PNS and play a less significant role in the CNS, within sensory neurons significantly more of them are found in the smaller sensory neurons which are likely to be nociceptors (Cardenas *et al.*, 1995). This fairly specific distribution of N-type VGCCs makes them excellent targets for analgesic drugs. Inhibitors have been shown to relieve severe pain in humans, and to relieve allodynia in a rat neuropathic pain model, without having any effect on acute pain (Scott *et al.*, 2002). The analgesic effects of opiates are due to mu opioid receptor activation having an inhibitory effect on N-type VGCCs (Schroeder *et al.*, 1991). However, due to the presence of some N-type VGCCs in the CNS higher doses of drugs targeting these channels can have unpleasant side effects such as nausea, disorientation, hallucination and agitation, requiring dosage to be carefully monitored (Penn and Paice, 2000).

#### **1.1.3.4 Voltage Gated Potassium Channels**

Voltage-Gated Potassium Channels (VGKC) are not vital for the action potential in sensory neurons but play a role in the properties or “shape” of it. When opened, VGKC take the membrane potential towards that of potassium, resulting in a decrease in excitability, making them a potential regulator of hyperexcitability in hyperalgesia. There is a vast variety of VGKC but they can be loosely categorised according to the number of transmembrane domains they possess. The classic VGKC has 6 transmembrane domains and it is in one of nine subgroups, these channels are often known as delayed rectifiers, as they alter the potassium current with a small delay after voltage activated opening (Gutman *et al.*, 2003). Of particular interest in pain is K<sub>v</sub>1.4 which has been shown, by immunohistochemistry, to be expressed in small diameter neurons that also

express  $\text{Na}_v1.8$  (Rasband *et al.*, 2001). This evidence, in combination with electrical physiological properties of this channel, makes it a candidate for a delayed rectifier in nociceptive neurons.

The two other structural classes of VGKCs have either two or four membrane domains, the former are inward rectifiers that are constitutively open and determine the resting membrane potential. Little is known about the four-transmembrane VGKC, although, interestingly, there is some evidence for their regulation by acidity in the gut (Holzer, 2003); further research may show that these channels play a key role in nociception.

There are also potassium channels that are gated by factors other than voltage. Within the six transmembrane groups, two channels have been documented that play a key role in excitability. These M and H channels regulate resting membrane potential in response to regulation by G-protein coupled receptors. M channels are present on sensory neurons where they can be opened via activation of muscarinic acetylcholine or peptide stimulated GPCRs (Jentsch, 2000) this suggests they may play a role in pain regulation an idea that has been supported by Passmore *et al* (2003) who demonstrated that inhibiting M channels leads to a significant decrease in hyperalgesia in rats following intraplantar carrageenan injection.

#### **1.1.4 Sensory Receptors**

##### **1.1.4.1 Transient Receptor Potential Cation Channels**

A family of channels called transient receptor potential cation channels (TRP) includes many of the temperature sensitive channels that are all in the Vanilloid subgroup (TRPV). Perhaps the best documented of these channels is TRPV1, a cation specific channel expressed in sensor neurons and activated both by heat and capsaicin, a chemical found in hot chilli peppers. TRPV2 and TRPV4 have also been demonstrated to be activated at noxious temperatures (Tominaga and Caterina, 2004, Green, 2004).

There are also channels activated at cooler temperatures for example TRPM8 (the metastatin subgroup), which is also activated by menthol and TRPA1 (containing ankyrin repeats) a receptor sensitive to both cold and mechanical stimulation. Cold pain differs from pain caused by heat in that the threshold of cold nociceptors is much further from normal mammalian body temperature. All A-fibres respond to temperatures below 0°C and cold sensation is generally mediated by A $\delta$  fibres that have a tonic baseline activity at room temperature and demonstrate a significant increase in firing on cooling (Tominaga and Caterina, 2004, Green, 2004).

The TRPA1 receptor has an activation threshold of 17.5°C and has been found to colocalise with receptors responding to heat and capsaicin, suggesting a role for this receptor in the burning sensation associated with some cold pain (Story *et al.*, 2003). Some TRP channels can also be regulated by endogenous mediators; TRPV1, for example, is activated by protons and TRPV2 and TRPV4 by cell volume. Along with capsaicin many other exogenous substances can modulate TRP channels leading to burning (e.g. alicin) or cooling sensations (e.g. menthol). The properties of the major TRP channels are summarised in Table 1.

#### **1.1.4.2 Acid-Sensing Ion Channels**

Acidosis is a common sign of tissue damage and inflammation and, as previously mentioned, TRPV1 can be activated by protons. However, the proton activation threshold of this channel is relatively high by which point irreparable damage may have occurred. Acid-sensing ion channels (ASIC) are activated at much lower proton concentrations and have been demonstrated to play a key role in pain sensation. ASICs are members of the voltage-insensitive, amiloride-sensitive epithelial Na<sup>+</sup> channel/degenerin family of cation channels and are encoded by three different genes ASIC1, ASIC2 (BCN1) and ASIC3 (DRASIC) (Foulkes and Wood, 2008). An individual channel is formed by two subunits which may be hetero or homomeric, ASICs 1 and 2 have two splice variants each adding to the potential functional differences.

Channel	Activation Temp	Endogenous Modulators	Exogenous Modulators	Tissue Distribution
TRPV1 (VR1)	>43°C	Protons Anandamide	Capsaicin (chilli) Allicin (garlic) Piperine (black pepper) Camphor	Sensory neurons Bladder
TRPV2 (VRL-1)	>52°C	Cell volume		Sensory neurons Brain Bladder Spleen
TRPV3	~31-39°C		Eugenol Thymol Carvacrol Camphor	Sensory neurons Hypothalamic neurons Keratinocytes
TRPV4	>27°C	Cell volume		Sensory neurons Kidney Keratinocytes Hypothalamus Hair cells Merkel Cells
TRPM8 (CMR1)	~ 8-28°C		Menthol Eucalyptol	Sensory neurons Prostate epithelia
TRPA1 ANKTM1	<17°C		Isothiocyanates e.g.: Mustard oil Wasabi Horseradish	Sensory neurons

**Table 1 A summary of the most widely studied TRP channels and their properties.** For reviews see (Jordt *et al.*, 2003) and (Tominaga and Caterina, 2004).

At least 5 subtypes of ASIC have been described, ASIC 3 is expressed in large and medium diameter neurons where it has been shown to have a transient response to stimulation by relatively mild acidosis, there is mounting evidence for a role for this channel in mediating cardiac pain in ischaemic heart disease (Immke and McCleskey, 2001a). Under normal physiological conditions  $\text{Ca}^{2+}$  blocks the pore of ASIC3, lactate released in ischaemic muscle has been demonstrated to sensitise this channel to acid at higher pH by decreasing the extracellular  $\text{Ca}^{2+}$  concentration (Immke and McCleskey, 2001b).

#### 1.1.4.3 Mechanoreceptors

Sensory neurons also express receptors that are activated by mechanical stimulation leading to their physical distortion by bending or stretching. Such receptors are present in many areas throughout the body where they play key roles in maintaining homeostasis through regulation of skin contraction, distension of digestive tracts, blood vessels and the bladder as well as sensing both noxious and innocuous touch. Mechanoreceptors are unmyelinated axon branches populated with mechanosensitive ion channels which open in response to changes in membrane tension. Different classes of nociceptive neurons mediate different forms of mechanical stimulus; A $\alpha$  fibres regulate proprioception, A $\beta$  fibres touch and low pressure and A $\delta$  and C strong mechanical.

Skin is subjected to a large variety of stimuli, which can differ in mode and intensity, requiring a range of mechanoreceptors sensitive to different forms of energy. Electrophysiological methods have been highly effective in locating mechanoreceptors and identifying their properties. As well as an immediate response to a stimulus receptors also vary in the way in which they respond to persistent stimuli; receptors which respond quickly but stop firing even if the stimulus continues are described as rapidly adapting and those that maintain a response during a sustained stimulus are known as slowly adapting.

The specific receptors involved in mechanosensation are difficult to identify but tests can be done on isolated neurons to identify their electrophysical properties in response to varying mechanical stimuli, which ultimately may lead to identification of specific receptors. Ion channels have been identified in *Escherichia coli* bacteria, which open in response to osmotic pressure causing stretching of the cell envelope (Sukharev *et al.*, 1993), and potassium channels have been identified in mammalian cells that may play a similar role.

Finally, channels known primarily for their role in sensing chemical stimuli have also been implicated in regulating the response to mechanical stimulation. ASIC and TRP channels have been demonstrated to respond to specific mechanical stimuli in electrophysiological experiments. It is also likely that physical deformation of cell membranes will result in the release of chemical messengers such as ATP that can act on specific receptors and also modify the excitability of other receptors (Julius and Basbaum, 2001).

### **1.1.5 Chemical Stimuli and Sensitisation**

Tissue injury leads to sensitivity as a protective mechanism to prevent further injury and allow healing; a similar response is seen following an infectious insult. This sensitivity is mediated by the release of factors from both neuronal and non-neuronal cells. This milieu of inflammatory mediators is often referred to as the “inflammatory soup” (Dray, 1995, Julius and Basbaum, 2001). The ligands within the inflammatory soup exert their effects by acting directly on ion channels or indirectly via second messenger systems, some such as 5HT and ATP can target both.

The purpose of these substances is to provide protection to the damaged tissue and kill off any invading infectious particles. This is achieved by increasing blood flow to the region via vasodilation, by increasing the influx of macrophages and fibroblasts to the area by chemoattraction and by sensitising sensory neurons to encourage protection of the damaged region to allow time for healing. Of primary interest here, is the effect of this soup on the sensitivity of nociceptors and some of the key players are discussed below.

C-fibre activation results in the release and retrograde transport of a variety of inflammatory substances, including CGRP and substance P, which contribute to increased neuronal excitability, vasodilation and capillary permeability. An increase in substance P and damage to tissue results in the release of further mediators including  $K^+$ ,  $H^+$ , acetylcholine, histamine and



bradykinin (Bk) which in turn increase the sensitivity of high threshold mechanoreceptors.

Endogenous inflammatory mediators can mediate the nociceptive response by direct action on nociceptors or activation of inflammatory cells, which in turn release nociceptor regulating substances such as cytokines, chemokines and nerve growth factors. Sensitisation of nociceptors can also result in an increased response to baseline levels of endogenous ligands leading to symptoms such as hyperalgesia and allodynia.

The bidirectionality of sensory axons allows the rapid spread of neurogenic inflammation via nerve impulses and retrograde transport of substance P to the periphery. This can quickly sensitise non-injured/involved neurons contributing to primary and secondary hyperalgesia.

#### **1.1.5.1 ATP**

The nucleoside, adenosine and its derivatives adenosine monophosphate (AMP), adenosine diphosphate (ADP) and adenosine triphosphate (ATP) are often released from cells, particularly platelets, following tissue injury. Adenosine can directly activate nociceptors and administration of it and its phosphates has been demonstrated to cause pain in human and animal models, reviewed in Hamilton and McMahon (2000). ATP mediates its effects via purinoceptors ( $P2_x$ ) expressed on peripheral and DRG neurons

ATP is released by damaged cells and activated nociceptors. Sensory neurons express several  $P2_x$  receptors whose activation leads to increased activity and the subsequent release of more ATP resulting in a positive feedback loop. Of the  $P2_x$  receptors expressed,  $P2_x3$  is the most studied as it is specifically expressed on small diameter neurons within the dorsal root and trigeminal ganglia (North, 2004).

#### **1.1.5.2-Hydroxytryptamine (5-HT)**

Serotonin (5-HT) can directly activate nociceptors and potentiate pain initiated peripherally. Following activation by inflammatory mediators mast cells release platelet activating factor, which in turn results in the release of 5-HT from platelets.

5-HT can depolarise sensory neurons via direct activation of the 5-HT<sub>3</sub> receptor, a ligand-gated ion channel. 5-HT<sub>3</sub> has been shown to be expressed by both small non-peptidergic neurons in the periphery and Gabaergic neurons on the dorsal horn and spinal cord, indicating a significant role for this receptor in pain regulation. This suggestion has been borne out by evidence from gene knock-out studies where mice lacking 5-HT<sub>3</sub> demonstrated a significant decrease in persistent pain in the setting of tissue injury (Zeitz *et al.*, 2002). Moreover the same authors demonstrated that this 5-HT-mediated effect is not due to an involvement in inflammation or oedema but via direct activation of a specific subgroup of non-peptidergic sensory neurons and activation of neurons within the CNS.

#### **1.1.5.3 Protons**

Acidosis often results from tissue damage, ischaemia or inflammation and the degree of acidosis correlates with pain and discomfort. In cultures, DRG neurons demonstrate both transient and sustained excitatory responses when exposed to acidic solutions. In similar experiments, around 50% of isolated C-fibres produced sustained currents (Krishtal and Pidoplichko, 1980, Reeh and Steen, 1996). As previously mentioned, several receptors are implicated in mediating responses to protons including TRPV1 and ASICs. The TRPV1 channel can be directly activated by protons and is likely to play a role in the burning pain associated with acid stimulation (Bevan and Geppetti, 1994, Caterina and Julius, 2001). Interestingly, protons can also act as allosteric regulators of TRPV1, decreasing the thermal activation threshold to below body temperature; this latter form of regulation may be significant in thermal hyperalgesia as a result of tissue damage (Tominaga *et al.*, 1998).

Both electrophysiological and behavioural experiments support this idea; C-fibres or DRG neurons cultured from mice in which the TRPV1 gene has been deleted demonstrate significantly decreased sustained activity at pH 5 compared with wild type and TRPV1 knock-out animals do not develop thermal sensitivity following inflammatory insult (Caterina *et al.*, 2000, Davis *et al.*, 2000).

#### **1.1.6 Neuropathic pain**

Neuropathic pain differs from pain that is the result of tissue injury or inflammatory insult since it occurs as a result of injury to neurons within either the central or peripheral nervous systems. This kind of injury can be associated with sensory loss but, perhaps more significantly, often results in an increase in stimulus evoked and spontaneous pain. Spontaneous pain following peripheral nerve injury is thought to result from ectopic firing of these nerves leading to a barrage of firing within the spinal cord. This type of pain, which is difficult to treat and often becomes chronic, will be discussed in further detail in Chapter 2.

#### **1.1.7 Drugs in the periphery**

NSAIDs (non-steroidal anti-inflammatory drugs) are well known for their anti-inflammatory action via inhibition of prostaglandins (PG); they are however ineffective in the treatment of neurogenic inflammation which acts via the distinct mechanism described above. Steroids have been shown to be effective in some cases of neurogenic pain, their mechanisms are unknown although there is evidence that they act on histamine, 5-HT, Bk and PG.

Local anaesthetics may prevent higher sensitisation in neuropathic pain, suggesting they may have significant practical applications in the prevention of post-operative pain. Capsaicin has also been demonstrated to have some effect in the treatment of neuropathic pain as it depletes substance P from the nerve terminals decreasing sensitivity. This treatment has been

particularly effective in treating neuralgia where nerve pain occurs in the absence of nociceptor stimulation (Woolf and Chong, 1993).

Peripheral nerve damage can result in ectopic firing, where a nerve fires spontaneously or abnormally. Anticonvulsant drugs such as carbamazepine which are traditionally used to treat abnormal firing in the brain in conditions such as epilepsy have been used with some success in treating neuropathic pain in conditions such as post hepatic and trigeminal neuralgia (Cross, 1994).

Burning pain that occurs as a result of C-fibre damage can be treated with low-dosage local anaesthetics. Such drugs act by inhibiting sodium channel activation and therefore decreasing firing, since C-fibres are unmyelinated they are susceptible to much lower doses of anaesthetic and their myelinated neighbours will remain unaffected (Stracke *et al.*, 1992).

#### **1.1.8 Hyperalgesia and Allodynia**

Hyperalgesia and allodynia are symptoms of both acute and chronic pain; they describe an increased nociceptive response to sensory stimulation. Hyperalgesia is an increased response to stimuli that are normally painful, i.e. a reduced thermal threshold leading to a burning sensation at body temperature, and allodynia is a painful response to normally innocuous stimuli i.e. light brushing of the skin. Primary hyperalgesia affects the damaged tissue itself and secondary hyperalgesia is increased sensitivity in the surrounding area. In a normal acute pain response these symptoms encourage increased protection of the damaged tissue to allow healing without further damage; however, they can become problematic where they persist after healing or occur where there is no apparent injury.

### 1.1.8.1 Primary hyperalgesia

Heat hyperalgesia occurs as a result of sensitisation of nociceptors, there is a correlation between subjective pain ratings of humans following a burn injury and recordings from monkey nociceptors following the same injury (LaMotte and Campbell, 1978). The type of sensitisation seen varies greatly depending on the injury, the tissue type and the nociceptors involved. Primary mechanical hyperalgesia is loosely divided into two categories, stroking hyperalgesia or allodynia, where stroking with a swab elicits a painful response, and punctate mechanical hyperalgesia, where a pin prick stimulus results in an increased pain response. Stroking hyperalgesia/allodynia occurs as a result of sensitisation of low threshold mechanoreceptors in A-fibres and an increased painful response to pin-prick stimulation occurs due to sensitisation of C-fibres.

Progressive tactile hypersensitivity may be associated with inflammation where inflammatory mediators increase the excitability of neurons and receptors in their vicinity (Ma and Woolf, 1997, Ma and Woolf, 1996b). Hyperalgesia and allodynia are a significant example of the plasticity of the nervous system due to changes in the properties of neurons and their receptors. These changes can occur at all levels of the nervous system and are specific to the type and duration of insult.

In the periphery, functional diversity occurs following tissue damage leading to an increase in painful responses. One way in which sensitivity might be increased is through lowered threshold of nociceptive nerves causing them to respond to lower level stimuli. Heat and mechanical injury do not directly decrease the threshold of activation but an inflammatory insult does and, depending on the type and extent of burn or mechanical injury, some secondary inflammation may occur and contribute to primary hyperalgesia (Davis *et al.*, 1993). In some cases the increase in response of nociceptors is not due to a decreased threshold but an increased response to suprathreshold stimuli (Cooper *et al.*, 1991). An increase in the receptive field of individual neurons also contributes to the hyperalgesic state by leading to

more frequent and less specific activation. Finally, there is evidence for ectopic firing in nerves within damaged tissue leading to increased spontaneous firing in the absence of any stimulus (Djouhri *et al.*, 2006).

Another significant contributor to hyperalgesia is a loss of central or descending inhibition from the brain and spinal cord. This will be discussed in the following section.

## **1.2 CNS Processing of noxious stimuli**

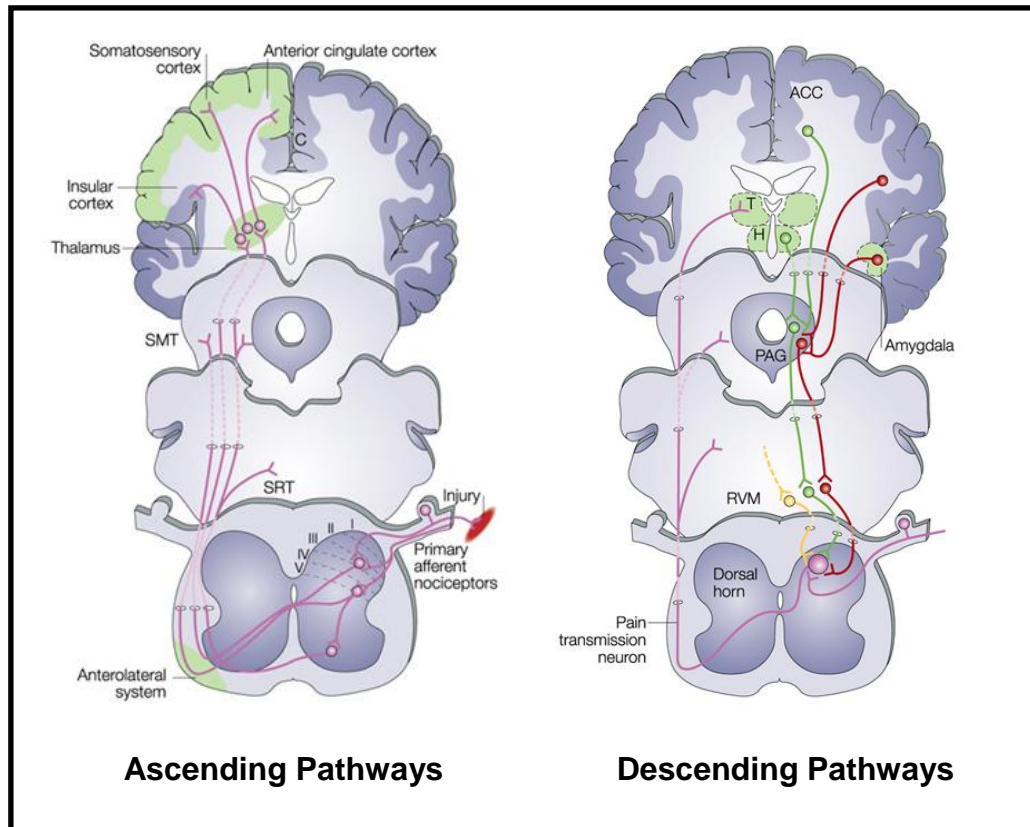
Pain differs from other sensory modalities in that it is not merely a physical response to external events; it is an internal perception of tissue damage. In general, senses are exteroceptive, informing us of the nature of the environment we find ourselves in. Pain as a description of the location and 'type' of pain is also exteroceptive, however, it can also be considered an interoceptive sense, such as thirst or hunger. Pain also differs from other senses in that it is considered to be real even in the absence of physical damage. This is not the case in vision, for example, where a response in the absence of a physical stimulus is considered to be a hallucination.

### **1.2.1 First Pain Synapse and Spinal Cord**

First order primary afferents for innocuous touch and painful sensation synapse in different parts of the CNS. Tactile afferents for fine touch, vibration and proprioception travel up the spinal cord and synapse with second order neurons within the medulla. These secondary afferents cross to the contralateral side of the brainstem and form the medial lemniscus pathway to the thalamus.

Primary afferent nociceptive and thermal sensory neurons from the periphery project to the spinal cord where neurons of different types synapse in distinct laminae; the A $\delta$  fibres synapse in laminae I and V, A $\beta$  fibres in laminae III, IV and V and C-fibres in laminae I (peptidergic) and II (non-peptidergic). The secondary afferents then decussate and ascend the spinal cord on the

contralateral side to the initial stimulus. Lamina II of the spinal cord contains a number of excitatory inter-neurons which are suggested to be involved in the development of chronic pain (Malmberg *et al.*, 1997).



**Figure 3 The Primary ascending (left) and descending (right) pathways.**

In the ascending pathway activated primary afferent neurons synapse within distinct laminae of the dorsal horn of the spinal cord releasing glutamate and other excitatory modulators. The subsequently activated second order neurons cross to the contralateral side of the spinal cord where they terminate in the brainstem and thalamus synapsing with third order neurons projecting to higher brain areas.

The descending pathway can be activated by external and internal modulators and motivational states. These pathways can be both excitatory (red) and inhibitory (green). The limbic system plays a key role in descending regulation with areas such as the anterior cingulate cortex (ACC), the hypothalamus (H) and the amygdala all synapsing in the periaqueductal grey (PAG) of the midbrain. Within in the PAG top down information is consolidated before transmission to the rostral ventral medulla (RVM) which then modulates pain messages from the dorsal horn. Messages within the RVM can also be modulated by 5-HT in a state-dependent manner.

Diagrams taken from: Nature Reviews Neuroscience 5, 565-575 (2004)

### **1.2.1.1 Dorsal Horn neurons**

The secondary neurons within the pain pathway have their cell bodies within the dorsal horn and axon terminating in the thalamus. They are broadly divided into two categories: Wide dynamic range (WDR) neurons which respond to gentle stimuli and increase in response when the stimulus increases and nociceptive specific neurons (NS) which only respond to noxious stimuli (Besson and Chaouch, 1987).

Wide dynamic range neurons are in laminae IV, V and VI, where they receive input from A $\delta$  and C fibres. Damage to neurons in the periphery leads to increased responses in these neurons, this is known as “wind-up” where a consistent peripheral stimulus will result in an increased response from the neurons of the DRG. If this continues the DRG neurons become over stimulated and themselves exhibit ectopic firing. WDR neurons also receive input from the viscera and play a key role in referred sensations (Fields, 1987).

The nociceptive specific neurons are primarily with lamina I of the spinal cord, where a significant amount of input is received from C and A $\delta$  fibres, and are specific to the sensory-discriminative aspect of pain. Receiving only noxious input these neurons are also sensitised by repetitive stimulation. Complex neurons projecting from laminae VII and VIII receive convergent input from visceral receptors, often bilaterally. Within the DRG of the spinal cord a single cell body may receive input from more than one axon in the periphery and often from more than one nerve. This combined with the release of substance P in the area means more than one site may be excited. This increased sensitivity and excitation within the DRG contributes to secondary hyperalgesia and “wind-up” (Hunt *et al.*, 1987).



### **1.2.1.2 Ascending Pathways**

Both WDR and NS neurons decussate the spinal cord in the anterior white commissure and then ascend the anterolateral quadrant (ALQ) of the spinal cord. The ALQ comprises many ascending pathways including the spinothalamic tract (STT), which terminates in the thalamus, and the spinoreticular tract (SRT), which terminates in the dorsal horn.

Of the STT neurons ascending to the thalamus approximately 50% comprise WDR neurons, 30% are NS and 10% are activated by deep tissue stimulation (Besson and Chaouch, 1987). Around 2% of ascending axons are thought to be activated by innocuous touch. Within the thalamus nociceptive STT terminate in two main regions, those from laminae I and V, which mediate the sensory-discriminative response, within the lateral nuclei and neurons from laminae I, IV and VI, which mediate the affective-motivation response, within the medial nuclei (Cross, 1994).

SRT neurons ascend the ALQ to the reticular formation, a primitive part of the brain involved in the regulation of basic functions necessary for life, the bulbopontine group of neurons ascend to nuclei within the pons and medulla and the mesencephalic to the periaqueductal grey, superior colliculus and the nucleus cuneiformis. This primitive pathway is crucial to the affective motivational aspect of pain and is the origin of descending pathways (Besson and Chaouch, 1987, Cross, 1994).

There are other smaller ascending pathways which contribute to pain perception and are likely to be involved in longer term persistent pain, including projections to SII and the insular cortex. These will be discussed further in the next chapter.

### **1.2.1.3 Segmental Modulation**

The pain signal from the two descending pathways is modulated in two different ways, segmental and descending modulation. The segmental modulation within the spinal cord is primarily inhibitory and occurs by two methods. The primary neurons for non-nociceptive sensation also have their cell bodies within the DRG and the secondary neurons in this pathway have an inhibitory effect on nociceptive activity, hence rubbing can help alleviate pain (Bini *et al.*, 1984). Transcutaneous electrical nerve stimulation (TENS) is a commonly used method of pain relief which also works by stimulation of these non-nociceptive neurons leading to a decrease in pain sensation which can last for several hours (Johnson and Martinson, 2007).

The second method of inhibition within the dorsal horn is via a network of inhibitory interneurons which may have their effect through endogenous opioid release as opioid receptors have been demonstrated in this region. Substance P receptors are also found here suggesting excitatory regulation may also occur in this region under certain circumstances contributing to wind-up and sensitisation (Basbaum and Fields, 1984).

### **1.2.2 Thalamus**

The thalamus is commonly considered as a relay station for sensory information (Herrero *et al.*, 2002). It receives input from both the peripheral and central nervous systems and its primary role is to integrate information for use by the higher cortices.

The major nociceptive output from the spinal cord to the thalamus is from laminae I and V which contribute to the spinothalamic and spinoreticulothalamic tracts. The thalamus plays a key role in integrating information from all of the senses before it is transmitted to the cortex, collating both sensory and discriminative signals. The thalamus is not only an “information exchange” it has a significant effect on the regulation of pain symptoms, particularly the affective-motivational responses; lesions in this area lead to

chronic pain indicating that it may play a role in inhibitory regulation (McGlone and Reilly, 2010, Urban and Gebhart, 1999).

Sensory information from the face and mouth is a slight exception to this rule as primary neurons from this region synapse at the trigeminal ganglion. Secondary neurons then decussate and synapse in the thalamus joining the same pathway as information from the rest of the body.

### **1.2.2.1 Thalamocortical Projections**

Third order neurons make up the final stage of the ascending pathway projecting from the thalamus to the cortex (Besson and Chaouch, 1987). The lateral thalamic nuclei projects to SI and the intralaminar and medial nuclei to the anterior cingulate cortex (ACC). The former being involved in the representation of the pain or the sensory-discriminative aspect and the latter the affective-motivational response, the anterior cingulate cortex is also activated when it is apparent that other people are in pain and may be implicated in the empathetic response (Cross, 1994, Ohara *et al.*, 2005).

### **1.2.3 Cortical Regulation**

The primary sensory cortex, SI, is the most well-studied area in the brain relating to sensory perception. It is made up of a detailed map of the body known as the Penfield's Homunculus (see Figure 10), specific regions correspond to specific body areas.

In both animal and human studies of nociception the degree of SI activation is not as significant as might be expected. Other areas of the cortex are activated, often bilaterally, suggesting a role in the affective-motivational response as well as the sensory-discriminative (Ohara *et al.*, 2005).

In acute pain human studies, areas that are activated include, the anterior insular cortex, SII, associative parietal cortex, anterior cingulate and the prefrontal and supplementary motor cortices. Recent research suggests that

pain may be the result of the activation of several brain areas at once, the so called “pain matrix” or “association cortex” (Ohara *et al.*, 2005). The areas of the brain activated and the order in which this happens may help differentiate between the wide variety of pain sensations experienced.

The cingulate cortex is part of the limbic lobe, forming a collar around the corpus collosum, it is divided into the anterior and posterior cingulate cortices. There is little or no evidence for the involvement of this region in the discriminative-sensory response to nociception, but there is considerable evidence that it is involved in the affective-motivational response. As part of the limbic system the cingulate cortex is implicated in the regulation of emotional and empathetic responses, it receives significant input from the thalamus where nociceptive information is processed.

Electrophysiological studies in animals have shown that the ACC receives nociceptive input to large receptive fields (Sikes and Vogt, 1992, Yamamura *et al.*, 1996). The size of the fields further suggests that the role of the ACC is unlikely to be sensory-discriminative since they are too large to be precise. Abolishing activity in this region by application of opioids or creating a lesion has an anti-nociceptive effect in mice, whereas enhancing responses here using glutamate or electrical stimulation results in hyperalgesia. Moreover, these responses can be blocked by anaesthetising the rostroventrolateral medulla (RVLM). Experiments in the medial prefrontal cortex, which is rostral to the ACC, have demonstrated opposite effects, which also appear to be mediated by descending inhibitory pathways (Lee *et al.*, 1999, Calejesan *et al.*, 2000).

Experiments using withdrawal responses and other measurements of hyperalgesia/allodynia do not directly measure affective-motivational responses. In a study using “place preference”, Johansen *et al.* (2001) found that formalin-treated rats with lesions of the ACC had no altered acute response to formalin, but had a reduced avoidance of the chamber in which the formalin injection was received. Injection of the cholinergic antagonist, scopolamine, into the ACC prior to a painful stimulus resulted in a decrease

in pain after the stimulus, however when the antagonist was given after the stimulus there was no decrease in nociception. This suggests that the ACC is significant in the “memory” of pain.

#### **1.2.4 Descending Regulation**

Nociception is not merely a “one way street” with information from the periphery terminating in the CNS and resulting in a sensation; descending regulation plays a key role in regulating nociception and can be both excitatory and inhibitory. The majority of descending regulation comes from the cortices, however, the spinal cord, dorsal root ganglia and periaqueductal grey (PAG) can also regulate pain responses without cortical modulation. Several neurotransmitters are known to regulate descending pathways including, adrenaline, NA, 5HT and opioids (Basbaum *et al.*, 2009, Cross, 1994, Ohara *et al.*, 2005).

The ventrolateral orbital (VLO) region of the frontal cortex is thought to be a significant part of the pain matrix as it is activated by cutaneous and visceral nociceptive stimuli. Morphine injection into the VLO increases initial tail flick latency and analgesia in neuropathic rats in a naloxone-reversible fashion, and similar effects are seen following application of anaesthetic or lesioning of the region (Al Amin *et al.*, 2004).

Studies on the rostral area of the insular cortex (RAIC) have demonstrated that this area can contribute to both increases and decreases in nociceptive thresholds. Increasing the levels of the inhibitory neurotransmitter, gamma-aminobutyric acid (GABA), causes an increase in nociceptive thresholds, blocking descending inhibition abrogates this response. Interestingly, the RAIC can also mediate its effects without activation of the lower centres; if GABA is increased and the GABA<sub>B</sub> receptor subsequently blocked, there is a decrease in nociceptive threshold, an effect which is reversed by blocking activity in the amygdala. Of particular note is the fact that these areas of the limbic system can exert antinociceptive effects without activation of the descending inhibitory system (Ohara *et al.*, 2005).

Descending projections from these higher centres terminate in the PAG where information is collated and then transmitted to the RVM. The RVM is rich in 5-HT and supplies a tonic inhibitory stimulus, as such a decrease in 5-HT in this area may result in an increased pain response. Noradrenaline also contributes to tonic inhibition in a manner that is selective to dorsal horn nociceptive neurons. Intrathecal administration of noradrenaline acts via  $\alpha$ -adrenoceptors to have an analgesic effect. Stimulation of the nucleus raphe magnus within the RVM results in release of both 5-HT and noradrenaline. Acute stress can decrease the pain response via an increase in NA and 5-HT. However, it is known that, in long-term stress and depression, pain can increase, and that this may be due to the depletion of these neurotransmitters.

The locus coeruleus (LC) of the pons also projects noradrenaline-releasing neurons that synapse in the dorsal horn. The LC is stimulated by the hypothalamus resulting in inhibition of nociceptive transmission in the spinal cord. The LC can also exert negative feedback on the hypothalamus via the release of NA.

These recent discoveries about the importance of monoamines in descending regulation of pain have led to the use of antidepressants such as amitriptyline HCL and selective serotonin re-uptake inhibitors in the treatment of chronic pain with varying degrees of success. Electrical stimulation by placement of electrodes in varying parts of the descending pathway is also a common treatment.

Opioid receptors are also found in the periphery, dorsal horn and brain stem. The receptors of the brain stem are the primary targets for endogenous opioids and are important for the regulation of the internal antinociceptive system; lesions in the area can inhibit endogenous opioid effects.

Within the PAG and ventromedial nucleus of the hypothalamus (VMN) there are two classes of descending neurons; excitatory or “on” neurons and inhibitory or “off” neurons. The former, directly or indirectly increase

nociceptive transmission and are inhibited by both NA and opioids, whereas the latter, decrease nociceptive transmission and are excited by opioids.

Descending inhibition is not the only form of endogenous analgesia. Recent research has brought to light the significance of inter-cortical regulation. Depending on the areas involved, this form of regulation can not only alter the degree of nociception but also the quality of the pain experience. For example, lesions of the frontal cortex do not decrease the sensory perception of a painful stimulus but can alter the amount of “unpleasantness” experienced (Talbot *et al.*, 1995, Ploner *et al.*, 1999).

### **1.2.5 Psychological Symptoms**

As previously mentioned, pain is not merely a physical symptom, by its very definition it is “An unpleasant sensory and emotional experience” (Merskey and Bogduk, 1994) involving many complex psychological and social factors.

Acute pain is not associated with many long-term psychological symptoms, a severe injury may be associated with psychological problems such as post-traumatic stress disorder (PTSD), but in these cases the pain itself is not usually the cause of the issues. Chronic pain, however, can have a significant impact on the psychological health of sufferers and conversely, a person’s psychological state can make a huge contribution to the development of chronic pain conditions. There is a significant link between depression and chronic pain, particularly with conditions such as fibromyalgia (FM), complex regional pain syndrome (CRPS) and chronic fatigue syndrome (CFS). Anxiety is also a significant problem in chronic pain patients; this is unsurprising since anxiety and depression frequently go hand in hand. Anxiety can occur for many reasons, for example, patients may become afraid of going out or doing exercise for fear of making their condition worse.

Pain can lead to disordered sleep and when a person’s sleep patterns have been significantly disrupted over a long period of time this can lead to longer-term sleep problems. Lack of sleep is detrimental to physical and

psychological health, often resulting in a vicious downward spiral of symptoms.



# Chapter 2

## 2 Introduction: Chronic Pain

The plastic nature of the nervous system allows for a non-dependable relationship between intensity of stimulus and perception of pain by alterations in neurons and receptors at all levels. Under normal conditions this plasticity is helpful, enabling the best response for the circumstances, for example an increase in sensitivity following tissue damage in order to allow the sensitive tissues to heal. Unfortunately this plasticity, the very thing that makes the nervous system so efficient, can also lead to inappropriate responses where pain is felt in response to innocuous stimuli or with no stimulus at all. As discussed earlier, chronic pain is usually defined as pain that does not result from disease or physical trauma, or that persists after physical symptoms have healed (Merskey and Bogduk, 1994). In practise the definition of pain as chronic depends very much on the condition involved and the expected healing time of said condition. There are also myriad chronic conditions in which pain is a predominant symptom, including, fibromyalgia (FM), complex regional pain syndrome (CRPS), arthritis, cancer, back pain, migraine, shingles and trigeminal neuralgia to name but a few.

### 2.1 Chronic Pain Conditions

The main physical symptoms of chronic pain, hyperalgesia (exaggerated response to normally painful stimuli), allodynia (painful response to normally non-painful touch) and spontaneous pain occur as a result of central sensitisation. In chronic pain the affective-motivational elements of the pain response become far more prevalent than in the acute response contributing to some of the most disturbing and intractable symptoms of chronic conditions.

The effects of chronic pain are far reaching, not only is there a significant effect on the physical and emotional wellbeing of the individual sufferer, the financial burden of healthcare and employee absence has a major impact on

the western economy. Estimations of the prevalence of chronic pain in developed countries range from 17-46% of the general population (Elliott et al., 1999, Blyth et al., 2001, Eriksen et al., 2003, Jakobsson, 2010a). This apparently wide range can, in part, be explained by the differing classifications and methods used in individual studies, with those at the lower end of the spectrum only considering pain going on for six months or longer as chronic and using medical records or phone interviews versus, those at the higher end classifying as chronic pain lasting for three months or longer and using self-completed written questionnaires. Even taken at the absolute lowest estimate of 17% of the general population it is clear that chronic pain is a significant problem for a large proportion of people in the western world. Chronic pain patients are up to five times more likely to seek medical help than non-sufferers making it a significant drain on health service resources (Elliott et al., 1999). This combined with its impact on individual physical and psychological health, as well as socio-economic wellbeing, makes it a highly significant problem facing the community today. Chronic low back pain, for example, accounts for 12.5% of all sick days in the UK and cost over £11 billion in 2000 (Frank, 1993, Maniadakis and Gray, 2000).

More detailed examination of the epidemiological statistics of chronic pain has provided increasing evidence of the significant personal and economic burdens of this condition. Chronic pain has been demonstrated to correlate with increasing age although some studies have shown that it peaks in middle age (45-59) and then decreases slightly in later life (Blyth et al., 2001, Elliott et al., 1999, Eriksen et al., 2003, Jakobsson, 2010b). There is also significant evidence to suggest that women suffer more frequently with chronic pain than men do and this has been supported in animal studies of male and female sex hormones (Manson, 2010). It should also be taken into account, however, that women are more likely to report and seek help for pain and as such there may be some bias in the statistics. The financial impact of chronic pain on both the individual and the economy is also high, with the condition being significantly associated with sufferers taking disability benefit, being unemployed and requiring long term support from health care systems, indeed, one study demonstrated that chronic pain sufferers have

twice as many days off due to illness than non-suffering controls and are up to seven times more likely to quit their job due to ill health. There are also significant social factors relating to chronic pain, lower levels of completed education, rented versus owned accommodation, unemployment and receipt of government benefits have all been significantly associated with chronic pain (Eriksen et al., 2003, Jakobsson, 2010a, Elliott et al., 1999, Blyth et al., 2001).

### **2.1.1 Complex Regional Pain Syndrome (CRPS)**

Complex regional pain syndrome (CRPS) is a condition which results in abnormal swelling, hypersensitivity and a significant amount of pain in the affected part of the body. It usually begins in an individual limb and can be triggered by injury, stroke or surgery, however, it often appears for no apparent reason. The condition is divided into two subtypes CRPS type I, where there is no causative nerve damage and CRPS type II where the symptoms can be related to some obvious nerve lesion. Previously, CRPS has also been known as reflex sympathetic dystrophy (RSD) and causalgia; it is thought to be related to several disorders including fibromyalgia (FM) and chronic fatigue syndrome (CFS).

The symptoms are many and varied but certain criteria have been developed for the diagnosis of the syndrome (Birklein, 2005). The most widely accepted criteria for diagnosis is that of the International Association for the Study of Pain (IASP) where symptoms must include; spontaneous pain and hyperalgesia, which is not limited to the territory of a single peripheral nerve and oedema, altered blood flow or abnormal sudomotor activity, although this is constantly evolving (Bruehl *et al.*, 1999, Reinders *et al.*, 2002, Stanton-Hicks *et al.*, 1995, van de Beek *et al.*, 2002) and is widely considered to be too general for accurate diagnosis given the variety of other symptoms commonly associated with the disease including:

- Changes in skin temperature, which can differ greatly from the contralateral limb
- Increased sweating
- Changes in skin appearance, blotchiness, change in colour
- Changes in hair and nail growth
- Motor disability, problems moving the affected body part

The order of presentation of symptoms and their relative severity varies from patient to patient but most report all of the above symptoms at some stage of their disease. Figure 4 shows examples of affected limbs demonstrating some of these symptoms.



**Figure 4 Examples of typical CRPS limbs.** Note the extensive swelling and oedema in the affected limbs. The foot also shows increased sweating and alterations in skin texture and colour compared with the healthy limb

More recently many unusual symptoms have come to light that are less physiological in nature and could in fact be considered to be psychological in their origin (Galer and Jensen, 1999, McCabe *et al.*, 2003a, McCabe *et al.*, 2003b). For many years such symptoms have been ignored or gone unnoticed as they were thought to be completely unrelated to the condition. However it has now become apparent that such symptoms are part of the CRPS condition and affect the majority of patients at some time. These symptoms, which could be considered to be part of the affective-motivational pain response, include:

- Altered visuospatial awareness
- Referred sensations
- A feeling of alienation towards the affected limb/neglect like symptoms
- Emotional lability
- Altered sleep and dreams
- Problems with memory and concentration

The body of research into these symptoms is growing and similarities between CRPS and other chronic pain conditions have become apparent (Forderreuther *et al.*, 2004, Juottonen *et al.*, 2002, Maihofner *et al.*, 2006, McCabe *et al.*, 2003a).

### **2.1.2 Chronic Low Back Pain (CLBP)**

Low back pain is one of the most common chronic pain complaints in western society affecting 70-85% of adults at some point in their lifetime of these around 5% go on to develop long term pain with an estimated 30% of the population suffering from the chronic form of the condition at any given time (Andersson, 1997, Frank, 1993).

Depression has been strongly linked with chronic low back pain (CLBP) (Meyer *et al.*, 2007, Reid *et al.*, 2003) and there may be many reasons for this, chronic pain is obviously a risk factor for depression, depression also tends to lead to a more sedentary life which, as mentioned above, has a negative effect on recovery from back pain. Furthermore, inflammation with the CNS has been linked with depression (Maes *et al.*, 2009) and is also known to contribute to central sensitisation in chronic pain (see section 2.2.1).

MRI scans of CLBP sufferers are abnormal when compared with healthy controls and metabolic changes have been seen that are similar to those seen neurodegenerative disease (Grachev *et al.*, 2000).

### **2.1.2.1 Treatment of low back pain**

Although previously rest was frequently prescribed for back pain recent evidence points towards early mobilisation along with effective analgesia as the treatment with the best potential outcome (Jasmin *et al.*, 2003). One mechanism by which this may work is through inhibition of central sensitisation by analgesia.

Using fMRI scanning and ratings of spontaneous pain fluctuations Baliki *et al* (2006) identified a pattern of activity specific to CLBP, two differing activation profiles were observed: when spontaneous pain was rapidly increasing the insula, anterior cingulate and parietal cortices along with the cerebellum were activated. When subjects rated spontaneous pain as high an increase in activity in the medial prefrontal cortex (mPFC) was observed while less activity was seen in the amygdala and ventral striatum. When comparing the response to an acute thermal stimulus no difference in pattern of activity was seen between CLBP sufferers and controls. This work shows a specific profile of activity in this chronic pain condition and demonstrates the importance of investigating brain activation in a symptom specific context and not only in response to an acute nociceptive stimulus.

Further analysis of the results of Baliki *et al* (2006) demonstrated a correlation between reported pain intensity and mPFC activation to the extent that greater than 80% of pain intensity can be accounted for by mPFC activity. Furthermore activity in insula cortex correlates with the number of years suffering from chronic pain. These results show that there is a specific matrix of activation seen in CLBP regardless of the initial cause of the condition. Since activity in the mPFC and dorsolateral prefrontal cortex (DLPFC) is normally inversely correlated, atrophy in the DLPFC is likely to contribute to the increase in activity seen in the mPFC.

Acute pain results in a fairly consistent pattern of brain activation in both chronic pain sufferers and controls (Apkarian *et al.*, 2005) whereas, individual chronic pain conditions have been demonstrated to show specific patterns of

activity with a significant increase in the prominence of the prefrontal cortex vs. acute pain (Furukawa and Gouaux, 2003).

### **2.1.3 Fibromyalgia**

Mu-opioid binding has been shown to be decreased in fibromyalgia patients by PET binding studies (Harris *et al.*, 2007) and altered dopamine release has also been demonstrated in this condition (Wood *et al.*, 2007).

Fibromyalgia is a relatively common condition with an incidence of 35/100000 people. It primarily affects women over the age of 40 and has been linked to the menopause (Wolfe *et al.*, 1997). Fibromyalgics present with a number of symptoms but the most widely accepted diagnostic criteria is that of the American College of Rheumatology (Wolfe *et al.*, 1990) as listed below:

- Widespread chronic pain for at least three months:
  - In both sides of the body
  - Above and below the waist including axial skeletal pain
- Pain at eleven of eighteen specific tender points on digital palpitation with an approximate force of 4kg.

Sufferers often report a wide variety of other symptoms including, severe fatigue, insomnia and cognitive problems such as impaired concentration and memory issues.

#### **2.1.3.1 Mechanisms of Fibromyalgia**

Wallace *et al* (2001) investigated the cytokine profile in the blood of 89 fibromyalgia (FM) patients, 23 with a disease duration of under two years and 33 diagnosed as chronic sufferers i.e. with a duration of two or more years. It has been suggested that there are two types of FM with 50% of patients diagnosed recovering within two years, whereas 95% of chronic patients show no significant recovery over a two year period (Wolfe *et al.*, 1997). Serum levels of a number of cytokines and related factors were analysed

along with their stimulated versus spontaneous release from peripheral blood monocytes (PBMCs) using ELISA.

Of all the factors measured, no significant difference in plasma levels of IL-1 $\beta$ , IFN $\gamma$ , IL-2R, IL-10, TNF $\alpha$ , and IL-2 was demonstrated. However, FM sufferers had on average a 55% increase in serum levels IL-1Ra versus normal healthy controls and plasma levels of IL-8 were three times higher than those seen in controls, this effect was primarily due to levels in chronic sufferers. Spontaneous cytokine expression was measured after a 72 hour incubation of un-stimulated isolated PBMC, IL-6 and IL-1Ra expression was higher in FM PBMC than control but this effect was only significant in cells from chronic sufferers. Spontaneous IL-8 expression was low across all groups and no significant difference was seen in levels of this cytokine.

Isolated PBMC were stimulated with Lipopolysaccharide (LPS) in the presence or absence of phorbol myristate acetate (PMA) which is known to stimulate T-cell activation. Without PMA, LPS induced similar levels of IL-1Ra, IL-6, IL-8 and IL-2 in FM PBMC to those seen in controls. However when 1ng/ml PMA was added along with the LPS, a significant increase in IL-1Ra was seen in the chronic FM group compared with both the control and the acute FM group, IL-6 also showed a significant increase this time in both FM groups.

These results, which are mediated by T-cell activation, demonstrate altered levels of circulating cytokines in FM, an affect that increases with duration of the disease. IL-6 is associated with many of the symptoms seen in FM including fatigue, hyperalgesia and depression, and IL-1Ra with stress (Pillemer *et al.*, 1997, Sachs *et al.*, 2002). The increased response to PMA and LPS seen in FM patients indicates a hypersensitivity of the immune system which may result in an excessive response to injury or infection leading to some of the symptoms seen in this condition. Ultimately, this may lead to a vicious cycle of increased pain, depression and stress resulting in elevated cytokine levels that in turn result in more pain.



Further support for this theory comes from the work of Uceyler *et al* (2006) who measured protein and mRNA levels of a wide spectrum of cytokines in the peripheral blood of 40 patients with chronic pain. 26 of these patients fulfilled the American College of Rheumatology (ACR) diagnostic criteria for FM. They found sufferers to have a significant decrease in both gene and protein expression of IL-4 and IL-10 relative to normal healthy controls. It has been suggested that these cytokines have anti-inflammatory and analgesic effects and IL-4 has been demonstrated to play an intrinsic role in switching the immune response from the cellular to the humoral response. In animal studies pre-treatment with this cytokine has been shown to decrease the writhing response to intraperitoneal (i.p.) acetic acid injection and allodynia following sciatic nerve ligation (Kanaan *et al.*, 1998, Vale *et al.*, 2003). This decrease in hyperalgesia may be due to the up-regulation of opioid receptors by IL-4. IL-10 has also been demonstrated to reduce hyperalgesia, although the mechanism by which this occurs is not yet known (Kanaan *et al.*, 1998, Vale *et al.*, 2003).

A reduced level of circulating anti-inflammatory cytokines would lend support to the positive feedback theory suggested above. Although FM is almost certainly the result of dysregulation of many systems it seems likely that a disproportionate response from the immune system plays a significant role in this disease.

#### **2.1.4 Chronic Fatigue Syndrome**

Chronic fatigue syndrome (CFS) is characterised by long-term severe fatigue combined with myalgia, cognitive disturbances, low grade fever and disturbed sleep. There is a known link with CFS and depression and it was long considered to be psychological in origin. However many of these symptoms also point towards a possible immunological or neurological cause.

Lange *et al* (1999) demonstrated significant abnormalities in the brains of a subset of CFS patients who had no history of psychiatric disorder but did demonstrate impaired cognitive functioning. The abnormalities were primarily

seen in the frontal lobes as small punctate hyper-intensities. These differences were seen in almost half of the patients (47.6%) with no psychiatric history. Of significant interest is that these differences were only evident when patients were divided according to psychiatric diagnosis, only 16.7% of patients with diagnosed psychiatric disorders and 10.5% of healthy controls had abnormal scans of the frontal lobe. This leads to the suggestion that there may be two subgroups of CFS sufferers, those whose disease is in some way the result of a psychiatric disorder and those with a disease of organic origin.

Building on this work, Natelson *et al* (2005) investigated cytokine and protein levels and the number of white blood cells in the cerebrospinal fluid (CSF) of 44 CFS patients. They found 30% of patients to have abnormal protein levels and/or increased numbers of white blood cells when compared to laboratory standards, all controls demonstrated normal levels. This subgroup of patients had a lower rate of current depression than those with normal CSF providing some support for the suggestion that their illness is organic and not psychological in origin. Of the 19 cytokines investigated 11 were identified at some level in CSF and 3 were found to show significant differences between groups. Granulocyte macrophage colony stimulating factor (GM-CSF) was found to be lower in CSF subjects compared to control, IL-8 was higher in patients with sudden onset CSF than control or gradual onset patients and IL-10 was higher in patients with abnormal CSF when compared with other groups. GM-CSF is important in the regulation of macrophage and granulocyte populations and its down-regulation in the presence of IL-8 and IL-10 can lead to a decreased response to certain bacterial infections (Hamilton, 2002, Lee and Rikihisa, 1996). In addition IL-10 has been shown to inhibit GM-CSF dependent monocyte survival and macrophage generation (Hashimoto *et al.*, 2001). Taken together these results provide support for the hypothesis that in some cases CFS is the result of a disorder of the immune system leading to neurological dysfunction.

Stress is a known factor in the regulation of the immune response. The hypothalamo-pituitary-adrenal axis (HPA) is the central mechanism for

regulation of the stress response that ultimately controls circulating levels of steroid hormones such as cortisol. One suggestion is that the dysregulation of the immune response in conditions such as CFS may be the result of decreased activity of the HPA axis leading to increased cytokine release in stress. To investigate this possibility Gaab *et al* (Gaab *et al.*, 2005)(Gaab, Rohleder, Heitz, Engert, Schad, Schurmeyer, & Ehler 2005) tested to see if a period of psychosocial stress altered the immune response to challenge by LPS. Subjects were exposed to two stressors, a fake job interview and a mental arithmetic task, immediately after this, and at several subsequent time points, blood and saliva samples were taken. As a measure of HPA axis activity, blood and saliva levels of cortisol and ACTH were obtained. To investigate the immune response whole blood was incubated with LPS overnight and cytokine levels measured by ELISA. CFS subjects had significantly lower ACTH levels than controls, however, their stress response did follow the expected pattern and levels of cortisol released were not significantly different to those of controls. In response to the immune challenge, numbers of leucocytes increased in the blood of both groups and there were significant differences in the stimulated cytokine production.

Contrary to initial expectations CFS patients showed decreased levels of both TNF $\alpha$  and IL-6 in the stimulated cytokine response following stress, exactly the opposite to the response seen in controls. There was no significant relationship between this difference and levels of cortisol suggesting that steroid regulation of cytokines was not the cause. These results lend further support to the theory that there is an altered immune response in CFS and also suggest a heightened sensitivity to glucocorticoid regulation in this condition. The immune system and the HPA axis are closely integrated and dysregulation of one often leads to problems with the other, these results suggest altered responsiveness of both systems in CFS.

## **2.2 Mechanisms of Chronic Pain**

As previously mentioned, different pain modalities show differing patterns of activation within the peripheral and central nervous systems, this is true of

both acute and chronic pain. Chronic pain is thought to be the result of central sensitisation which can occur at the level of the spinal cord and higher up in the brain. Multiple mechanisms are thought to contribute to sensitisation including inflammation, cortical reorganisation and wind-up.

The situation is further complicated by the fact that many chronic pain conditions contain several components, low back pain, for example, can result from muscle, nerve, joint, or bone injury or a combination of these things. Teasing out the precise cause or even the major contributing factor to many chronic pain conditions is often practically impossible.

## **2.2.1 Inflammation in the CNS**

### **2.2.1.1 Cytokines**

Cytokines are chemical messengers of the immune response; they mediate inflammation and are released in response to injury or other physiological insult such as bacterial infection. They set off a cascade of events leading to inflammation, infiltration of macrophages and vasodilation. They are known to stimulate 'sickness behaviour', a group of symptoms including: fatigue, malaise, inability to focus, fever and decreased appetite. Following tissue damage, cytokines also stimulate growth and repair and as such they are vital for recovery and development. Until recently the CNS was considered to be protected from cytokines, and related molecules, by the blood brain barrier. It has been demonstrated however that glial cells within the CNS release a variety of cytokines in response to physiological insult (McMahon *et al.*, 2005).

The inflammatory cytokines are primarily known as mediators of the immune response via secondary messenger cascades in response to physiological insult. Recent work, however, has demonstrated an additional role for cytokines in the regulation of neuronal sensitivity and activity in response to tissue damage and inflammation (Hellstrom *et al.*, 2005, Hoheisel *et al.*, 2005). This regulation manifests itself in several ways, including changes in

response to stimulation, altered sensitivity to neuronal mediators and in some cases through direct stimulation of the nerve itself (i.e. by acting as a neurotransmitter). Several cytokines have been demonstrated to play a role in the development of allodynia and hyperalgesia in both chronic and acute pain and others have been shown to decrease these symptoms (Marchand *et al.*, 2005, Covey *et al.*, 2000, Ignatowski *et al.*, 1999)

Administration of cytokines stimulates hyperalgesia and allodynia in animal and human models (Covey *et al.*, 2000, Turrin *et al.*, 2001). Increased levels of circulating cytokines are seen in the blood and cerebral spinal fluid (CSF) of chronic pain sufferers and this had previously been demonstrated in FM, CFS and CRPS (Alexander *et al.*, 2006, Alexander *et al.*, 2005, Natelson *et al.*, 2005, Uceyler *et al.*, 2006, Wallace *et al.*, 2001). In animal models of pain, increased levels of cytokines have been demonstrated in the brain and specifically within the hippocampus (Turrin *et al.*, 2001). Intracerebroventricular (ICV) injection of cytokines causes increased pain behaviour in animals and subcutaneous injection stimulates hyperalgesia and allodynia in human subjects.

How and why cytokines have these effects is of great interest at present. The fact that they are involved in the development of a heightened pain state (allodynia/hyperalgesia) during injury and healing can be easily explained as a protective response to tissue damage. What is more intriguing is that these mediators seem to also be released in chronic pain, where there is not always obvious injury or tissue damage. This leads to the suggestion that such cytokines may themselves be mediators of chronic pain conditions. Altered cytokine expression has been demonstrated in several conditions associated with chronic pain including, FM, chronic fatigue syndrome and CRPS (Alexander *et al.*, 2006, Alexander *et al.*, 2005, Uceyler *et al.*, 2006, Wallace *et al.*, 2001).

### 2.2.1.2 Cytokines in the Hippocampus

The hippocampus is one of the most widely studied areas of the brain, it is known to be involved in learning, memory, spatial mapping and navigation, emotional responses and stress regulation. The hippocampus is also considered to be involved in the perception of pain and a role for cytokines in the regulation of hippocampal activity is suggested.

Ignatowski *et al* (1999) used chronic constriction nerve injury (CCI) as a model of chronic neuropathic pain in rats and investigated TNF $\alpha$  distribution within the brain, spinal cord and plasma using a WEHI-13 cytotoxicity assay. Levels of the cytokine were found to increase in both the spinal cord and hippocampus of CCI rats when compared with both control and sham operated animals. There was a significant increase in TNF $\alpha$  concentration in the hippocampus from day 4 to day 6, post CCI. This increase correlated with the development of thermal hyperalgesia as measured by the hot plate test. This indicates a direct role for TNF $\alpha$  in the development of neuropathic pain, a suggestion that is further supported by inhibition of hyperalgesia when antibodies to TNF $\alpha$  are given four but not six days post CCI.

Following on from this, these authors (Covey *et al.*, 2000) performed a similar experiment where changes in TNF $\alpha$  levels in relation to the noradrenergic response within the hippocampus were investigated. The  $\alpha_2$  adrenergic receptor is an auto-inhibitory receptor and its activation is associated with modulation of pain (Kontinen *et al.*, 1998, Schmitt *et al.*, 1974) and regulation of hippocampal activity (Curet and De Montigny, 1988, Unnerstall *et al.*, 1984). As previously, levels of TNF $\alpha$  were elevated in the brain and spinal cord of CCI animals, with most significant differences in the brain being within the locus coeruleus (LC) and hippocampus. Interestingly, levels within the LC increased by day four, whereas hippocampal levels were only significantly increased at day eight, again correlating with the development of hyperalgesia. Along with this increase in TNF $\alpha$ , an increase in inhibition of stimulated noradrenaline (NA) release was demonstrated, this inhibition was

stimulated by  $\text{TNF}\alpha$  and inhibited by idazoxan (an  $\alpha_2$  adrenoreceptor antagonist). These results, combined with the fact that the hippocampus receives the majority of its adrenergic input from the LC, suggest that  $\text{TNF}\alpha$  mediates pain behaviour by modulation of the  $\alpha_2$  adrenoreceptor activity within these areas.

Further support for a modulatory effect of  $\text{TNF}\alpha$  on the  $\alpha_2$  adrenergic receptor is provided by its role in regulating the effect of the tricyclic antidepressant desipramine (Ignatowski and Spengler, 1994). Desipramine is an NA reuptake inhibitor, in rats chronic (14 days) desipramine administration reversed  $\text{TNF}\alpha$  induced inhibition of NA release resulting in increased release. However, when the  $\alpha_2$  receptor is blocked with idazoxan this reversal is attenuated such that the percentage inhibition is greater than that seen in idazoxan treated controls.

These results suggest a role for  $\text{TNF}\alpha$  in the regulation of adrenergic activity within the hippocampus via modulation of  $\alpha_2$  receptor activity. The different effects seen over time and with desipramine administration suggest that long term exposure to altered cytokine levels may modify the NA response leading to altered processing of pain other cognitive input. This may explain some of the variances seen between acute and chronic pain states.

#### **2.2.1.3 Cytokines in CRPS**

Recent work (Alexander *et al.*, 2005) has demonstrated an increase in the levels of cytokines in the CSF of CRPS patients when compared with non CRPS controls. The cytokines measured were IL-6, IL-1 $\beta$  and  $\text{TNF}\alpha$  using ELISA. As well as clear increases in levels, it was noted that there was a large variance in the levels of cytokines in the CRPS samples compared with controls. This suggests that there may be a change in the expression of these molecules over the duration of the disease and it would be interesting to see if this was linked to variations in physical symptoms and/or time course of disease.

In comparison to the above affects seen in the central nervous system (CNS) the innate cytokine profile within the blood of CRPS patients has been demonstrated to be normal both at 'baseline' and in response to stimulation by administration of LPS or thrombin (van de Beek *et al.*, 2001). As would be expected due to the presence of excessive inflammation, an increase in cytokines is seen in the affected limb when compared with the unaffected limb (measured in blister fluid). It would also be interesting to see if there is a correlation between levels of cytokines and extent of inflammation, temperature changes and colour changes in the affected limb.

Taken together these results suggest a role for altered cytokine expression in the development and maintenance of chronic pain conditions and their associated symptoms.

#### **2.2.1.4 Chemokines**

Chemokines are a family of over 50 proteins which play an important role in regulating inflammation in response to tissue injury, infection and tumour growth. Their name is derived from their function as chemotactic cytokines inducing leukocyte migration. The effects of chemokines are all mediated via G-protein-coupled receptors, this family of seven transmembrane receptors reacts to molecules outside the cell to activate signalling cascades within (Melik-Parsadaniantz and Rostene, 2008).

Chemokines are, in general, promiscuous ligands binding to several different receptors. There are exceptions to this rule, notably the chemokine fractalkine (CX3CL1), which binds to one receptor only and this receptor, CX3CR1, binds only fractalkine making it a good potential target for specific intervention. Fractalkine was first identified in the CNS and was initially known as neurotactin (Trettel *et al.*, 2008). Under normal conditions this molecule is highly expressed on the extracellular surface of neurons, attached by a membrane spanning mucin stalk. Strong excitation of the neuron results in cleavage of this stalk and release of the extracellular domain as a potent diffusible signal to surrounding neurons and glial cells.



Within the CNS the CX3CR1 receptor is primarily expressed on microglia however it has also been reported to be expressed by neurons of the hippocampus. Pain is a strong stimulus to neurons of the spinal cord and has been demonstrated to result in release of fractalkine from these neurons and to increase the expression of the chemokine receptor in pain related regions (Baliki *et al.*, 2003, Clark *et al.*, 2007, Axen and Porath, 1966).

### **2.2.2. Neurodegeneration**

Apkarian *et al* (2004b) demonstrated, for the first time, that chronic pain is correlated with decreased grey matter in the brain, specifically within the prefrontal cortex and thalamus. This work supports the theory that chronic pain is a form of neurodegenerative disease in which alterations in brain structure and chemistry contribute significantly to both physical and emotional/psychological symptoms.

#### **2.2.2.1 Evidence from Animal Models**

As discussed in Chapter 1, of the ascending pain pathways, the spinothalamic tract (STT) is the well-documented, although there is evidence that the spinorecticular, spinomesencephalic, spinoparabrachial and spinohypothalamic tracts all transmit pain information to the higher centres of the brain. There is also growing evidence that some neurons project directly from the periphery to the brain. Recent studies have further broken down the type of information transmitted in the STT indicating that the lateral pathway is involved primarily in sensory discrimination and the medial, which projects primarily to the ACC in the PFC, in the affective motivational response to pain (Apkarian *et al.*, 2005). This work has further contributed to the theory that higher brain centres play a highly significant role in the regulation of chronic pain.

The evidence for anti-nociceptive descending modulation in acute pain is well documented (Basbaum and Fields, 1984). Interestingly, recent work has demonstrated the presence of descending pathways which have a pro-

nociceptive effect (Lima and Almeida, 2002). Evidence for the role of descending pathways in maintaining neuropathic pain states comes from two recent studies; Vera-Portocarrero *et al* (2006) demonstrated a role for the ventromedial medulla in the maintenance of neuropathic pain in a spinal nerve ligation model. Ablation of mu-opioid expressing cells of the ventromedial medulla, prior to surgery, using dermorphin-saporin resulted in allodynia and activation of the ipsilateral dorsal horn only at day three and not days five and ten as was seen in neuropathic controls. Furthermore, both pro- and anti-nociceptive cells have been identified within the rostroventro medulla (Carlson *et al.*, 2007), these cells, termed “on” and “off” cells by the authors, showed increased sensitivity allodynia-inducing stimulation when compared with sham and normal controls, no effect was seen on so-called “neutral cells”, which have no-response to thermal stimulation.

#### **2.2.2.2 Evidence from Human Studies**

Opiates exert their effects not only in the higher areas of the brain but also within the spinal cord and receptors are also present in the periphery. Human brain imaging studies have demonstrated a role for opiate receptors within the thalamus, basal ganglia and cortex to be directly related to the perception of both acute and acute painful stimulus (Zubieta *et al.*, 2003, Zubieta *et al.*, 2002) and the chronic pain of FM (Harris *et al.*, 2007).

#### **2.2.2.3 Supraspinal Regulation of Chronic Pain – Pain Memory**

As intimated above, the cortex does not simply represent the activity of the spinal cord in pain; it plays an active role in the processing and regulation of it. Cortical modulations in both animal and human models can modulate pain behaviour (Baliki *et al.*, 2003, Han and Neugebauer, 2005, Jasmin *et al.*, 2003, Johansen and Fields, 2004).

The IASP definition of chronic pain as discussed earlier, “Pain that persists beyond normal tissue healing time, which is assumed to be 3 months”, and later proposed definitions such as that of Merskey and Bogduk (Merskey and

Bogduk, 1994), “Chronic pain is a state of continued suffering sustained long after the initial injury has healed”, could be described in terms of memory. Chronic pain is a “memory” of the initial response to tissue injury which for some reason has become more permanent and is maintained by substantial reorganisation within the CNS. It has been suggested that fear conditioning contributes to this process, where chronic pain is acting as an unconditioned stimulus leading to increased negative associations with situations and environments as a result of being in near constant pain (Apkarian *et al.*, 2008). This idea is further supported by the increasing prominence in these conditions of the mPFC, an area of the brain that has, in rodents, been demonstrated to play a key role in Pavlovian fear conditioning and emotional and cognitive processes (Millecamps *et al.*, 2007).

Recently, attempts have been made to test this “memory hypothesis” pharmacologically using D-cycloserine (DCS), a partial agonist of the N-Methyl-D-aspartic acid (NMDA) receptor (Furukawa and Gouaux, 2003), which has previously been demonstrated to enhance learning and memory and facilitate the extinction of acquired fear responses through the development of new memories via NMDA receptor regulation. Millecamps *et al.* (Millecamps *et al.*, 2007) found that oral administration of DCS to rats with spared nerve injury resulted in a dose-dependent decrease in mechanical sensitivity which continued for weeks after cessation of treatment. Acute antinociception could be induced by direct infusion of DCS into the mPFC or amygdala by an NMDA dependant process, furthermore, DCS treatment significantly reduced fear avoidance behaviours in SNL animals. In the mPFC of SNL rats, expression of the NMDA receptor NR2B was down-regulated; however, this effect was reversed with repeated oral administration DCS. Taken together these results clearly demonstrate a role for plasticity in the brain in the regulation of pain behaviour and suggest that NMDA receptor modulation may be a key target for development of drug therapies.

Recent studies have increasingly demonstrated a key role for the limbic system in the modulation of pain behaviour and maintenance, in particular the anterior cingulate, insula and media-prefrontal cortices and the amygdala.

These limbic areas are all strongly interconnected and key in emotional and social processing. In a notable study Johansen and Fields (2004) demonstrated that the ACC is both necessary and sufficient to develop an aversive memory to a painful stimulus in a glutamate dependant fashion. This leads to the suggestion that pain processing may lead to an LTP like process in the brain, a hypothesis further supported by the up-regulation of the NR2B component of the NMDA receptor within the ACC following inflammatory injury (Wu *et al.*, 2005).

In a rat model of inflammatory arthritis, Bird *et al* (Bird *et al.*, 2005) found that synaptic plasticity of neurons from the central nucleus of the amygdala was inhibited in arthritic animals when the antagonist had no significant effect on normal transmission of control neurons. Furthermore the ACC, hippocampus and amygdala have been implicated in fear memory acquisition in a process that also specifically requires NMDA within the ACC (Malin and McGaugh, 2006, Zhao *et al.*, 2005).

These results demonstrate that NMDA receptors regulate pain transmission and undergo plastic changes to contribute to and perpetuate the persistent pain state by altering neuronal activity.

#### **2.2.2.4 Long Term Potentiation**

Long term potentiation (LTP) has been widely studied in the hippocampus as the cellular basis of learning and memory and cytokines are implicated in the regulation of this process. LTP is the strengthening of signalling at a synapse (i.e. the potentiation of the signal), it is measured electrophysiologically as an increase in excitatory post-synaptic potential (EPSP), which lasts for at least one hour. There are considered to be two phases or forms of LTP, the shorter term 'early phase' LTP which is protein independent and lasts for one to five hours and the longer term 'late phase' LTP. This latter phase results in a more permanent strengthening of the synapses by increased gene and protein expression whereas in the early phase the strengthening of signalling

is thought to be due to increased release of transmitters such as glutamate and the increased activity of these receptors.

Interleukin 1 (IL-1) has been implicated in the regulation of LTP, Ross *et al* (Ross *et al.*, 2003) investigated the role of endogenous and exogenous IL-1 in LTP in the mouse hippocampus. There are several members of the IL-1 family IL-1 $\alpha$ , IL-1 $\beta$  and IL-1 receptor antagonist (IL-1ra) these cytokines interact with two receptors IL-1 receptor 1 (IL-1RI) and IL-1 receptor 2 (IL-1RII) but signalling only occurs following binding with IL-1RI which results in the subsequent activation of a signalling cascade via association with IL-1 accessory protein (IL-1AcP). The inhibitory cytokine IL-1ra competitively binds IL-1RI but does not associate with IL-1AcP therefore not activating the cascade. These cytokines and their related factors are all known to be expressed within the hippocampus and other brain areas where IL-1 administration has been demonstrated to both stimulate and inhibit synaptic activity (Wang *et al.*, 2000, Zeise *et al.*, 1992).

Levels of endogenous interleukins were measured in mouse hippocampal slices at room (21-24°C) and physiological (34-36 °C) temperatures with significantly higher levels of both IL-1 $\alpha$  and IL-1 $\beta$  being expressed at the higher temperature. It is also interesting to note that IL-1 $\alpha$  was by far the more abundant of the two cytokines at physiological temperature. In order to stimulate LTP in the slices high frequency tetanic stimulation was used in a 'theta-burst stimulation' (TBS) paradigm, with five trains of fifteen 100Hz quadruple bursts. This resulted in significant potentiation of the EPSP, which lasted for over one hour. At room temperature, application of IL-1 $\beta$  prevented TBS-induced LTP, and this effect was abolished by co-administration with IL-1ra. When IL-1ra was administered alone LTP induction was not affected however at physiological temperatures IL-1ra inhibited LTP development in a concentration dependant manner. These results demonstrate a dual role for IL-1 in LTP, low levels being necessary and higher levels inhibitory.

In an effort to understand some of the processes behind this alteration in LTP, Hellstrom *et al* (Hellstrom *et al.*, 2005) investigated the membrane

properties of hippocampal neurons exposed to LPS in organotypic slice culture. Neurons exposed to LPS for seven days demonstrated similar resting membrane potentials and action potential properties to control cultures. LPS exposed neurons however, did show a significantly lower membrane resistance and while the action potential threshold was elevated, the frequency was decreased leading to a decreased probability of action potential firing. These authors also demonstrated a significant increase in IL-1 $\beta$  release at two days following the LPS exposure with levels returning to baseline after four days. A similar effect was seen with IL-6 expression; IL-6 expression is thought to be induced by increases in IL-1 $\beta$  however application of IL-1ra did not entirely abolish the LPS-induced increase suggesting another mechanism by which IL-6 release is stimulated. Interestingly, IL-1ra also decreased action potential frequency and when applied with LPS an additive effect was seen, suggesting LPS is altering action potential properties via an IL-1 $\beta$ -independent mechanism.

Using pharmacological inhibitors, it is possible to specifically measure either the Inhibitory post synaptic potentials (IPSPs) or EPSPs. No significant difference was seen in the EPSPs of previously LPS exposed versus unexposed neurons although there was a trend towards an increase. IPSPs, however, were significantly potentiated in treated neurons, an effect that was reversed when they were co-incubated with LPS and IL-1ra. This finding that GABAergic inhibition is enhanced in an IL-1ra-dependant manner, several days after LPS treatment, suggests that IL-1 $\beta$  may have a long term inhibitory effect on overall synaptic activity.

Initially this research appears to contradict the previously suggested role for IL-1 $\beta$  in regulating LTP as it demonstrates no significant difference in EPSP. However, as discussed earlier different concentrations of the cytokine have differing effects with a U-shaped dose response curve. A role of IL-6, independent of IL-1 $\beta$ , has also been suggested. It seems likely that time scales play an important role as Ross *et al* (2003) looked at the effects of one or two hours as opposed to several days. Prolonged chronic cytokine exposure may have longer-term effects on LTP production and memory,

whereas acute increases have transient reversible effects. It seems likely therefore, that longer-term exposure is changing the mechanisms of long-term LTP by altering gene and protein expression leading to overall inhibition of activity, whereas in the shorter-term, more temporary effects are occurring via modulation of ion channels and changes in neurotransmitter release.

## **2.3 Psychological Symptoms**

### **2.3.1 Emotional Decision Making**

Apkarian *et al* (2004a) examined emotional decision making capability in CRPS and CLBP and found both of these groups to be significantly impaired in this area when compared with healthy controls. Interestingly, although performance in CLBP was correlated with reported levels of pain at the time and modulated by sympathetic block, this was not seen in CRPS indicating that different brain mechanisms are involved in these two conditions.

## **2.4 *In vivo* Experiments**

As discussed above, cytokines have many effects at the cellular level and animal studies have also demonstrated significant behavioural effects. IL-1 plays a significant role in both spatial memory and passive avoidance conditioning but has no effect on non-spatial navigation as demonstrated by Yirmiya *et al* (2002) using two experimental paradigms, the Morris water maze (MWM) and the passive avoidance test (PAT). In the water maze, the rat's natural dislike of water and swimming ability are exploited to make it search for a hidden platform in a pool of water. In the spatial test, rats are given visual cues or landmarks and they must find and then remember the location of the platform in relation to these landmarks. In the non-spatial form of the test the platform is clearly marked and the animal merely learns that the mark represents the location of the platform.

In the PAT animals were given limited access to drinking water (30 minutes per day) and then placed in a cage where the water spout was slightly electrified. Following training and acclimatisation, the rats were given a shock

when they went to drink. The latency to return to the drinking spout on a following occasion was measured to see if the animal had 'learnt' to avoid the shock.

To test the effect of IL-1 on these learning paradigms rats were infused i.c.v. with either IL-1 $\beta$ , IL-1ra or saline immediately after each test. IL-1ra significantly inhibited learning in the spatial MWM but had no significant effect in the non-spatial version. In the PAT, IL-1ra-injected animals had significantly decreased latencies compared with controls and IL-1 $\beta$  treated animals significantly increased, both of these effects increased over time. These results demonstrate a significant role for IL-1 in memory processing and learning of both spatial and fear-conditioned events, a theory further supported using IL-1r knock out (IL-1 KO) mice.

Using the MWM along with a cage that provided a foot shock, spatial memory and fear conditioning were shown to be significantly attenuated in IL-1 k.o. mice (Avital *et al.*, 2003). Performance in the non-spatial MWM and an auditory conditioning task were, however, unaffected. *In vivo* recording in the dentate gyrus of IL-1 k.o. animals demonstrated increased paired pulse inhibition and LTP could not be generated in CA1 of the hippocampus in slice preparations. Together with the previous results this provides evidence for cytokine mediated changes in processing at the cellular level leading to changes in behavioural responses and specific forms of memory development.

## 2.5 Chapter Discussion

While identifying the areas of the brain that are activated in certain circumstances is of great importance, it is also becoming apparent that the temporal organisation of activation is of great significance. For example, in CLBP patients show an abnormal activation pattern in the resting state (Baliki *et al.*, 2008) and in post-hepatic neuralgia (PHN) a change in the temporal activation of specific brain areas in allodynia has been linked to the magnitude of pain (Geha *et al.*, 2007).



Unravelling the role of individual networks is further complicated by the fact that descending and ascending pathways commonly act in parallel and/or converge on the same areas making teasing out the role of one specific network all the more difficult (Bee and Dickenson, 2007).

From the brief discussion of research presented here, it is evident that cytokines play a significant role in the regulation of the CNS and that these CNS effects are closely correlated with physical and behavioural symptoms in both humans and animals. The experiments discussed here tend to concentrate on the effects of IL-1 $\beta$  and TNF $\alpha$  and these are indeed the mostly widely studied cytokines in this context. Although such work provides invaluable insight into the role of cytokines it is important to remember that the immune response involves a complex interplay between a huge number of such molecules with both inhibitory and stimulatory effects. Further work into the role of other cytokines is clearly needed to further support and understand the results presented here. The evidence for a precise role for altered cytokine expression in CRPS and other chronic pain states is far from conclusive. It is certain however, that sufferers of such conditions do have abnormal cytokine expression with a trend towards an increase in inflammatory cytokines and a decrease in the inflammatory molecules, this coupled with altered regulation of the HPA and endocrine axes could lead to many of the physical and cognitive symptoms described. It is also likely that the cytokine profile changes over the course of a disease ultimately predicting the severity and chronicity of the condition

## Chapter 3

### 3 Introduction: Methods

#### 3.1 Animal Models of Chronic Pain

The use of animals in the study of pain is controversial, it is not possible to replicate the human experience of these conditions precisely and as such the use of such models has been called into question, however, they can be considered a good starting point to improve understanding of pain mechanisms at the cellular and systems level. Many models exist that have been developed to represent the different types of pain experienced in human conditions as closely as possible. Pain can broadly be classified by its origin, either inflammatory or neuropathic, and its persistence, acute or chronic. As such animal models have been developed to reproduce these types of pain; recently elaborate models of lesser studied types of pain such as bone and cancer pain have also been developed.

##### 3.1.1 Inflammatory Pain Models

The most common cause of pain is inflammation which can occur as a result of tissue injury either by physical or chemical insult, many diseases that cause pain are also the result of inflammation e.g. rheumatoid arthritis. Inflammatory pain in animal models is usually induced by the injection of an inflammogen, the most common injection site in rodents is the hind paw which has several benefits over other areas in that it is easily accessible for measurements, can be easily seen in behavioural tests and can also be compared with a non-injected paw in the same animal (Morris, 2003, Larson *et al.*, 1986, Hargreaves *et al.*, 1988, Iadarola *et al.*, 1988) (see Figure 5). Other injection sites include i.p. injection as a model of visceral pain, injection into a joint to simulate arthritis or direct injection into a muscle for example the masseter muscle as a model of orofacial inflammation.



**Figure 5 An example of the footpad model of inflammatory pain.** The right hindpaw is noticeably swollen following intradermal injection of an inflammogen, in this case 0.1% CFA. The non-injected left paw remains unaffected and can be used as a within group control.

As well as location of injection, the choice of inflammogen is important when designing a pain model. Formalin is commonly used to induce an acute response lasting about one hour, there are two stages to the formalin response, in the initial stage, which results from direct activation of peripheral nociceptors, the animal keeps the injected paw lifted and does not use it to bear weight (Puig and Sorkin, 1996). This first phase lasts around five minutes after which the animal will begin to bear weight on the affected paw but can be seen to show other pain behaviours such as guarding and licking of the injected paw. This second phase is thought to result not only from persistent activation of peripheral nociceptors, as in the first phase, but also from increased excitability of the dorsal horn fibres (Bruehl *et al.*, 1999, Taylor *et al.*, 1995). The TRPV1 agonist, capsaicin, is also commonly used since it acts specifically on nociceptors it can be used to model neurogenic inflammation. Intradermal capsaicin injection produces hyperalgesia along with the classic “wheal and flare” reaction which lasts up to an hour (LaMotte *et al.*, 1991).

Other substances can be used to induce longer term inflammatory pain, some of the most commonly used are carrageenan, mustard oil, zymosan and complete Freund's adjuvant (CFA) (Hargreaves *et al.*, 1988, Iadarola *et al.*, 1988, Ma and Woolf, 1996a, Ma and Woolf, 1996b, Neumann *et al.*, 1996).

CFA is a solution of *Mycobacterium tuberculosis* emulsified in mineral oil, it acts as an immunopotentiator, stimulating the cell-mediated immune response. In animal models, specific injection of CFA can lead to a localised inflammatory response inducing inflammation within two hours of application showing an initial peak at six to eight hours with hyperalgesia, oedema and inflammation being present for more than three weeks (Raghavendra *et al.*, 2004, Stein *et al.*, 1988). Although this cannot really be considered a model of chronic pain, since it does not persist for many weeks or months, it is commonly called a "persistent pain model" and can be used to compare mechanisms of longer term pain with those seen in more acute models. The degree of oedema in this model has been demonstrated to correlate with behavioural symptoms of pain and immunological evidence of a cellular inflammatory response (Stein *et al.*, 1988, Nagakura *et al.*, 2003).

Carrageenan (CAR) is a mucopolysaccharide extract of the *Chondrus crispus* algae that is commonly used as a food additive. When injected into soft tissue CAR induces a rapid, non-immune, highly reproducible response which peaks at around three hours and returns to normal within 24 hours unless repeated applications are given (Morris, 2003). As such, the CAR model can be considered a model of "sub-acute pain" (Iadarola *et al.*, 1988, Morris, 2003).

There are many methods of assessing pain in animal models, one of the earliest, and still most commonly used, techniques is the paw withdrawal reflex, first reported by Hargreaves *et al.* in 1988 (Hargreaves *et al.*, 1988). In this method, a radiant heat stimulus is applied to the paw of the subject and the withdrawal time measured. A decrease in withdrawal time at a given temperature is considered to be evidence of hyperalgesia. A technique often

used alongside the Hargreaves method is the use of Von Frey hairs to measure the response to pressure, a decrease in paw withdrawal time in response to a given pressure is considered to be evidence of allodynia (Ren and Dubner, 1993, Kim and Chung, 1992).

The above measurements of pain response allow the animal to control the level of pain in response to a stimulus since they are able to remove their paw. Other methods can be used to get some measurement of spontaneous pain for example, the writhing response to visceral pain (Ness, 1999, Vyklicky, 1979), vocalisation (Wood *et al.*, 2007) and measures of behaviour such as lifting or guarding of an injured paw and autotomy (Kauppila, 1998, Tjolsen *et al.*, 1992, Olmarker *et al.*, 2002, Stein *et al.*, 1988).

### **3.1.2 Neuropathic Pain Models**

The most common method of inducing neuropathic pain in an animal model is through surgery, a specific nerve or group of nerves is damaged or cut and then the resultant behaviour compared with that of a sham operated animal. The three most common surgical models of neuropathic pain are; chronic constriction injury (CCI) where several small ligatures are loosely tied around one sciatic nerve (Frank, 1993), partial nerve ligation (PSL) where a single tight ligature is tied around a portion of the sciatic nerve (Seltzer *et al.*, 1990) and spinal nerve ligation (SNL) where a tight ligature is tied around the spinal nerves of one side of the body at L5 and L6 (Kim and Chung, 1992). Although these models all produce similar behavioural evidence of on-going pain, both evoked and spontaneous, there are subtle differences in the behaviours seen (Kim *et al.*, 1997). For example, SNL results in significantly higher mechanical allodynia than the other two models, lasting up to 20 weeks post-surgery, whereas, CCI, which has relatively lower mechanical allodynia results in more persistent on-going thermal hyperalgesia (Kim *et al.*, 1997). These behavioural differences may help contribute to the understanding of the mechanisms behind the symptomatology of various nerve injuries and neuropathic disorders.

### 3.2 Cell culture

Cell culture is a widely used technique in which isolated eukaryotic cells are grown *in vitro* under controlled conditions, the development and methodology of cell culture is closely related to tissue and organ culture where tissue and organ explants respectively are maintained *in vitro*. The correct conditions are needed for cell or tissue explants to survive *ex vivo*, nutrients are provided by specific growth media and temperature and humidity maintained within tight parameters using an incubator. Incubators are usually filled with a specific gas mixture to provide oxygen and help maintain the pH of the medium.

Some of the most significant early culture experiments were performed by Sydney Ringer in the late 19<sup>th</sup> century. Ringer demonstrated that it was possible to maintain a beating heart *ex vivo* if it was perfused with a specific salt solution; this became known as “Ringer’s Solution” and is still frequently used in medicine and research laboratories (Ringer, 1882).

Cell culture is a relatively cheap technique and allows for multiple experiments to be repeated under tightly controlled conditions. Experimental conditions can be easily manipulated to represent a variety of situations, for example, hypoxia or acidosis, and drugs or other reagents can be added at varying concentrations. This technique enables the use allows drugs or manipulations that would be impossible or unethical in animal or human models. Cells can normally be maintained in culture for several weeks allowing long term manipulations investigating a variety of time points to be carried out.

There are significant issues with cell culture experiments, the conditions in which cultures are maintained, no matter how carefully set up and preserved, are still far from those *in vivo*. Cells within an organism are in 3D whereas in culture they are often grown on a flat surface. Under culture conditions one cell line/type is commonly isolated and grown, in an organism many cell types exist synergistically. Depending on the cell type some of these issues

can be overcome for example by co-culture of more than one cell type or by organotypic slice culture.

### **3.2.1 Primary Neuronal cell culture**

Short term culture of neurons is relatively simple and useful for studying growth and development of cells and neurite initiation. In order to study fully formed neuronal cells and their function much longer culture times are necessary to allow adequate time for a complex network of connections to be made, this is somewhat problematic due to the delicate nature of this cell type and their highly specific requirements.

Most cell types can be fairly easily harvested from tissue by homogenisation and subsequent centrifugation. Obtaining a homogenous aliquot of viable neurons from brain tissue is not, however, without its difficulties; it is very difficult to separate the cells without damaging them since neurons grow together in a tightly knit complex network forming connections with many different cells. Brain tissue is not a homogenous mass of neurons, there are many other cells present, primarily the microglia and astrocytes, which, as well as providing immune protection, release growth factors and provide mechanical support for the neurons. Glial cells grow faster than neurons and are not so reliant on specific conditions; if the culture is contaminated with a significant number of such cells, they can negatively affect neuronal development.

The most common starting point for a neuronal culture is to use cells from an embryonic or newborn rat or mouse since few connections have been formed between cells at this stage meaning they are more easily separated, the majority of methods are based on that developed by Banker and Goslin (1998). Cultures are usually made from either cortical or hippocampal tissue depending on how they are going to be used. Hippocampal cultures allow for greater neuronal homogeneity since this area of the brain contains fewer glial cells than the cortex. It is, however, more difficult to dissect out this small part of the brain and since the amount of tissue it yields is relatively small, other

tissue, such as cortical tissue is often used where higher densities of neurons are needed, for example in western blotting (Meberg and Miller, 2003).

The need for specific growth factors from a variety of cells means that neurons do not grow well in long-term culture using standard media and serum. Standard growth media, such as DMEM, also contain substances such as cysteine and glutamine which affect neuronal regulation and ferrous sulphate, which is toxic to neurons. Serum is also problematic in that it encourages glial growth and proliferation, and although cytotoxic inhibitors can be used to reduce this problem they also have deleterious effects on neuronal growth (Wallace and Johnson, 1989).

*In vivo*, neurons obtain much of their essential nutrition and growth factors from surrounding glial cells, as such, the traditional method to provide these nutrients in culture is to either grow the cells on a bed of cultured glial cells or inverted over a glial culture or to feed them with glial conditioned medium (Banker *et al.*, 1998, Meberg and Miller, 2003, Bekkers and Stevens, 1991). The use of glial feeder cells is costly, time consuming, and can lead to contamination of cultures. To combat this issue, Brewer *et al* (Brewer *et al.*, 1993) developed a culture medium, Neurobasal, and a serum free supplement, B27, which contains essential factors to support long term growth on primary neuronal cultures while significantly inhibiting glial growth. Neurobasal and B27 are now widely available and have been demonstrated to support a wide variety of neuronal cultures (Brewer *et al.*, 1993, Brewer, 1995).

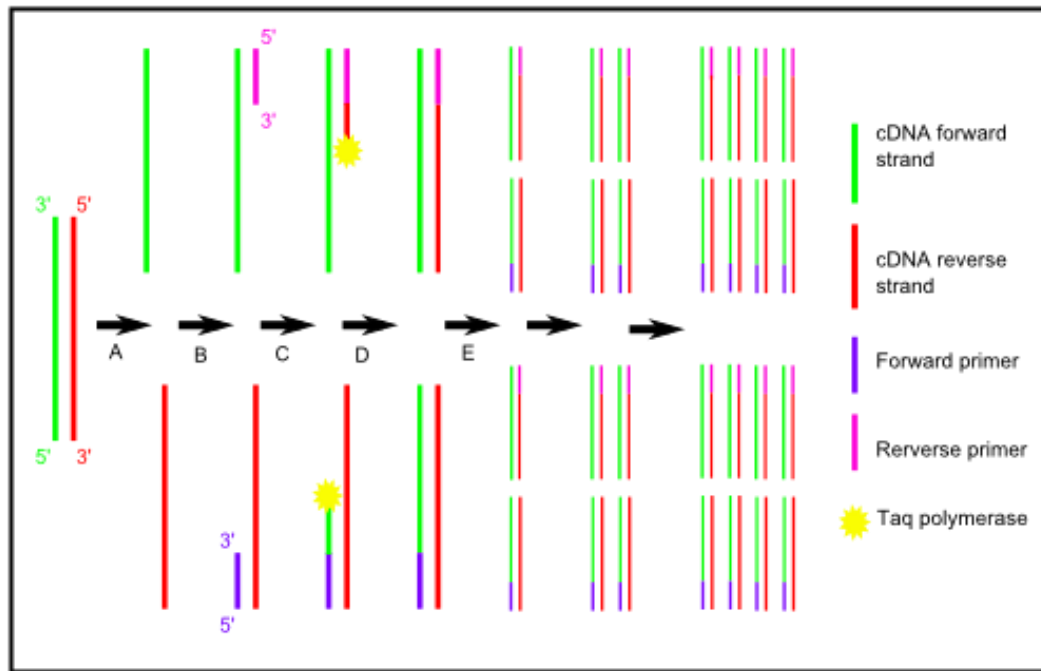


### 3.3 Molecular Biology

Molecular biology is, as the name suggests, the study of biology at the molecular level. It is used to break down whole body systems into their genetic and protein components and look at how these individual elements interact. In recent years molecular biology has taken a massive leap with the development of gene-knock-out animals and the mapping of the entire genomes of individual species including *Homo sapiens* in the Human Genome Project. Studies of molecular biology in healthy and diseased conditions can contribute greatly to the understanding of disease processes and often lead to the development of precisely targeted therapies.

#### 3.3.1 RT-PCR

The reverse transcriptase polymerase chain reaction or RT-PCR is one of the most frequently used molecular biology techniques, it allows many copies of a specific DNA sequence to be generated following the reverse transcription of RNA that has been previously isolated from a sample. Reverse transcription is the process by which single stranded RNA is transcribed into double stranded complementary DNA (cDNA) using a reverse transcriptase enzyme. A nucleic sequence of interest within the cDNA can then be targeted using specially designed primers. In order for the primers to bind to the cDNA a series of temperature changes are used, firstly the double stranded cDNA must be denatured at a high temperature into single strands. The temperature is then reduced to allow the primers to “anneal” to the DNA, this temperature can vary depending on the primer design and concentration as well as the cation concentration of the solution. Finally the DNA is extended from the primers using a polymerase enzyme the most commonly used being *Taq polymerase* which was named after the *Thermus aquaticus* bacterium from which it was isolated (*Chien et al., 1976*) (see Figure 6) for a summary of the entire PCR reaction. DNA increases exponentially through multiple cycles and is ultimately visualised using agarose gel electrophoresis with ethidium bromide.



**Figure 6 Schematic Drawing of the PCR Cycle.** cDNA from the RT reaction is first denatured at 94-96°C (A) and the subsequently separated strands annealed to reaction specific primers at ~60°C (B). The next step is elongation which occurs at 72°C, the activation temperature of *Taq polymerase* (C) resulting in two copies of the segment of interest (D). The cycle is then repeated multiple times resulting in exponential production of the product (E).

### 3.4 Immunological techniques

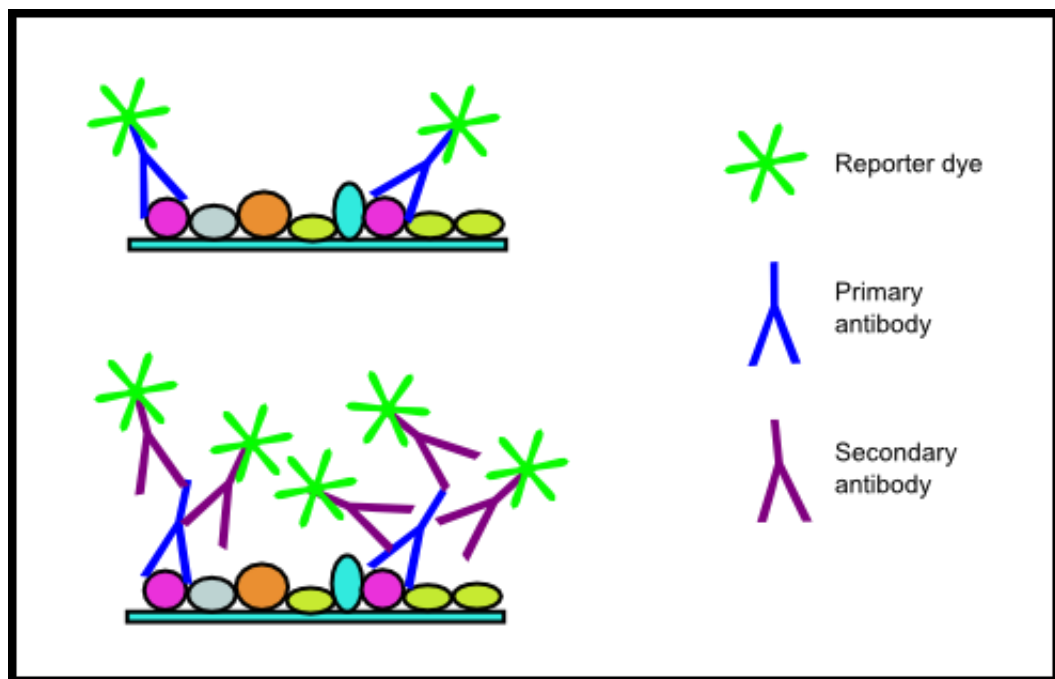
Immunology is the study of the immune system including antigens and their specific antibodies, this wide area of biomedical science has been harnessed for use in research and diagnostic laboratories for decades with new techniques utilising antibodies being frequently introduced.

#### 3.4.1 Immunohistochemistry and Immunocytochemistry

Immunochemistry is widely used to locate specific protein antigens within tissues and cells populations. One of the primary benefits of this technique is that it allows the location of antigens to precise areas within the sample, by using a mixture of two or more primary antibodies it is also possible to show colocalisation of different proteins.

The simplest method of Immunohistochemistry is the *direct method* using an antibody that is conjugated to a dye to show up the labelled regions. This

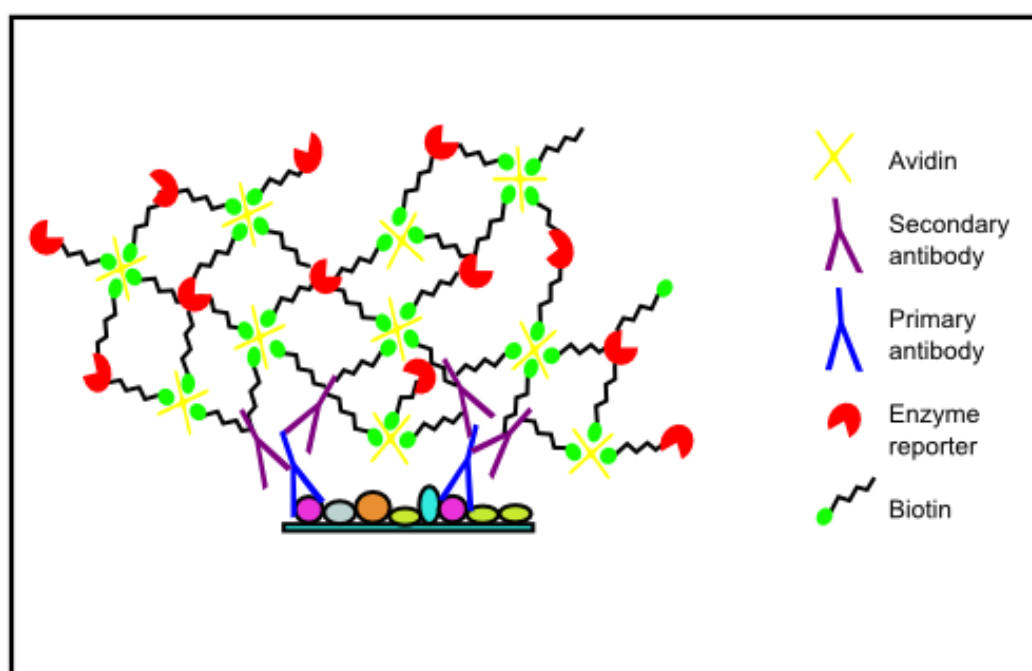
method is not commonly used, however, as it does not allow for any significant amplification of the signal and has little opportunity for optimisation. The more common *indirect method* was used here where a primary antibody, raised in a species other than the species of interest, is first applied to the sample. This is followed by a secondary antibody which is raised against the IgG of the species that the primary antibody was developed in (see figure 7).



**Figure 7 The two basic methods of immunological staining.** The *Direct Method* (top) in which the primary antibody to the antigen of interest is conjugated to a dye or fluorescent molecule. In the *Indirect Method* (bottom) the primary antibody binds to the antigen of interest and a secondary antibody raised against the IgG of the species the primary was raised in is added. The secondary antibody is conjugated to a dye and since multiple secondary antibodies can bind one primary molecule the signal can be amplified.

Two common methods of visualisation were used in this work, the first being immunofluorescence where the secondary antibody is conjugated with a fluorescent dye such as fluorescein isothiocyanate (FITC) and viewed under a fluorescence microscope. Fluorescent staining was typically used when more than one antigen was studied in the same sample as colocalisation can be very clearly seen in this method.

The second detection method used is the “Avidin: Biotinylated enzyme Complex” (ABC) method where the secondary antibody is labelled with a biotin molecule which had four binding sites for the vitamin avidin. Avidin has an incredibly high affinity for biotin and as such binding between these molecules is essentially irreversible. Following binding of the secondary antibody the ABC complex mixture is added which contains an enzyme for detection of product (see Figure 8).



**Figure 8 The ABC Staining Technique.** The primary antibody binds to the antigen of interest and a biotinylated secondary antibody is added. Following binding of the secondary antibody the ABC complex is added, each avidin molecule binds up to four biotin which in turn bind to enzyme reporter molecules. The complex is then visualised by addition of a substrate resulting in a coloured precipitate.

### 3.4.2 Hypoxyprobe

Hypoxyprobe or, Pimonidazole hydrochloride, is a bioreductive compound used to label hypoxic areas in tissue or cells. Under hypoxic conditions such compounds generate reactive metabolites which bind to cellular macromolecules permanently labelling them even if the area is subsequently re-oxygenated. Hypoxyprobe is a nitroaromatic compound, such compounds have been widely studied and extensively used as markers of hypoxia. In

1976 Varghese *et al* (Varghese *et al.*, 1976) used  $^{14}\text{C}$ -labelled misonidazole to target hypoxic areas in Chinese hamster ovary cells and KHT tumour cells.

Adducts form with thiol groups in proteins, peptides and amino acids under hypoxic conditions, i.e. where  $\text{pO}_2$  is less than 10mmHg. It has been demonstrated that this irreversible binding is dependant only on the degree of hypoxia and is not mediated by redox enzymes, NADH or NADPH levels. The oxygen dependence of misonidazole binding is close to that of radiation resistance and, as such, it can be used as a hypoxia marker in solid tumours.

The use of radioisotopes has limited clinical utility, particularly with the commonplace use of radiotherapy in cancer treatment and this led to the development of non-invasive assays using 2-nitromidazoles using PET, nuclear medicine analysis and magnetic resonance spectroscopy.

Following the success of *in vivo* techniques it became apparent that some form of marker at the cellular level would be highly desirable and an immunological method was developed using these compounds to pinpoint hypoxic regions within tissues and individual cells.

Antibodies were raised against protein adducts of reductively activated 2-nitromidazoles. A variety of markers have enabled the precise labelling and localisation of hypoxic regions as well as allowing for the investigation of colocalisation with other markers.

### **3.4.3 ELISA**

The enzyme-linked immunosorbent assay (ELISA) is an alternative method of utilising an antibody to detect as specific antigen. Unlike immunological staining techniques, they can give accurate measurements of the amount of antigen present and are therefore useful for quantifying responses to experimental manipulations. In order to visualise the ELISA reaction antibodies are bound to an enzyme which will result in some form of quantifiable signal such as fluorescence or colometric dyes. ELISAs are

usually performed in 96-well plates, to which the sample or cells of interest have been attached.

Prior to the development of ELISA, radioactivity was used to quantify the amount of bound antibody, these radioimmunoassays (Yalow and Berson, 1960) were both dangerous and time consuming so an alternative, quantifiable, marker of antibody binding was sought. Stratis Arameas and G.B. Pierce individually developed a method by which enzymes, such as peroxidase, could be linked to an antibody resulting in a measurable colour change on addition of a relevant substrate (Lequin, 2005). Since the ELISA relies on the antigen of interest being attached to a surface, the vessel being used must be rendered “immunosorbent” a method first demonstrated by Wide and Porath (1966) using the dextran gel “Sephadex”.

There are two forms of ELISA, the first is indirect where the sample is immobilised on the plate by adsorption to the surface. The plates are blocked using a non-specific protein to prevent any irrelevant binding of the antibody. Following blocking, the specific antibody is added and then visualised either directly if it is conjugated to an enzyme or indirectly via a secondary antibody. This form of ELISA is expensive and can be problematic for samples containing very small amounts of antigen as all proteins in the sample will bind to the plate potentially masking the antigen of interest.

In a sandwich ELISA the plate is pre-coated with an antibody against the antigen of interest, a blocking solution is applied to prevent non-specific binding and the sample is added. The plate must then be washed to remove any unbound antigen and a second antibody is added, sandwiching the antigen between two specific antibodies. This second antibody can be conjugated to an enzyme for detection or a secondary enzyme-linked antibody added. Sandwich ELISAs allow for detection of smaller amounts of antigen as only the protein of interest is bound to the plate.

## Chapter 4

# 4 Experimental: Questionnaire-Based Clinical Study

### 4.1 Introduction

Over time it has become apparent that as well as the myriad physical symptoms characteristic of CRPS there are also a significant number of bizarre phenomena which appear to be psychological or non-organic in origin. Many sufferers report increased pain in response to visual and audio stimuli and altered visuo-spatial awareness (Birklein, 2005, Sunderland, 1976). They also demonstrate emotional lability, referred sensations and an altered perception of their affected limb (Maihofner *et al.*, 2006, McCabe *et al.*, 2003a, McCabe *et al.*, 2003b). Until recently these disturbing symptoms went largely unnoticed with sufferers and healthcare professionals alike considering them to be unrelated to the condition. In consultations many patients were afraid to mention such symptoms due to fear that they would not be believed. Recent work however has shown these symptoms to play a significant role in this condition and they are now being considered as important areas in research, treatment and diagnosis.

As a great deal of evidence in this area is anecdotal from clinics and consultations the number of sufferers experiencing these symptoms is not clear. To this end, a questionnaire was developed to further investigate their prevalence within a population of CRPS sufferers. The questionnaire included questions on a wide range of symptoms and was completed by CRPS sufferers attending a national symposium of the support group RSD UK. Some of the more significant findings are reported here. These findings were also presented at the IASP 'Pain in Europe VI' conference 2006.

## **4.2 Materials and Methods**

### **4.2.1 Methods**

A questionnaire was developed to investigate the prevalence of a number of unusual symptoms seen in CRPS. The questionnaire was distributed at a patient support symposium (RSD-UK annual conference 2005) 52 questionnaires were considered suitable for further analysis, the majority of which were completed by the patients themselves although some were assisted by their carer. All of the subjects had been previously diagnosed with CRPS type I or II using the accepted IASP criteria. The questionnaire was developed and administered, by Professor David Blake of the RNHRD Bath, prior to the start of this work. All subsequent analysis was performed by the author of this thesis.

Data were collated using an Excel spread sheet and in most cases answers interpreted as 'Yes', 'No' or 'Sometimes' by the analyst to allow for comparison. Specific questions asked are detailed in the following sections. Not all subjects answered every question.

### **4.2.2 Subject Demographics**

N = 52

Age: range 13-78 (median 41)

Sex: 45 female, 7 male

Duration of disease: 0.5 – 32 years (median 5 years)

This is representative of a typical CRPS population with the majority of sufferers being post-menopausal women. Due to the nature of the support group a large proportion of the subjects were chronic sufferers who had lived with CRPS for many years.



## 4.3. Limb Perception

### 4.3.1. Introduction

One of the most striking observations in CRPS is that sufferers often feel removed or separated from their affected limb, sometimes to the extent that they do not feel it belongs to them and, in extreme cases, they want to have it removed. All too often, severe cases of chronic CRPS result in the amputation of the affected limb due to a combination of unbearable physical symptoms and the disownership or alienation of the limb. These symptoms are not unlike neglect and alien hand syndrome (AHS) sometimes seen victims of stroke and brain damage. Neglect patients have an impaired or lost ability to react to or process sensory stimuli presented in one hemispace following damage to the cerebral cortex (Farne *et al.*, 2004, Kikkert *et al.*, 2006). This can manifest itself in several ways including disownership of parts of the body contralateral to the damaged hemisphere and an inability to see or recognise objects presented in the corresponding visual field.

The questions asked (in reference to the CRPS limb) were:

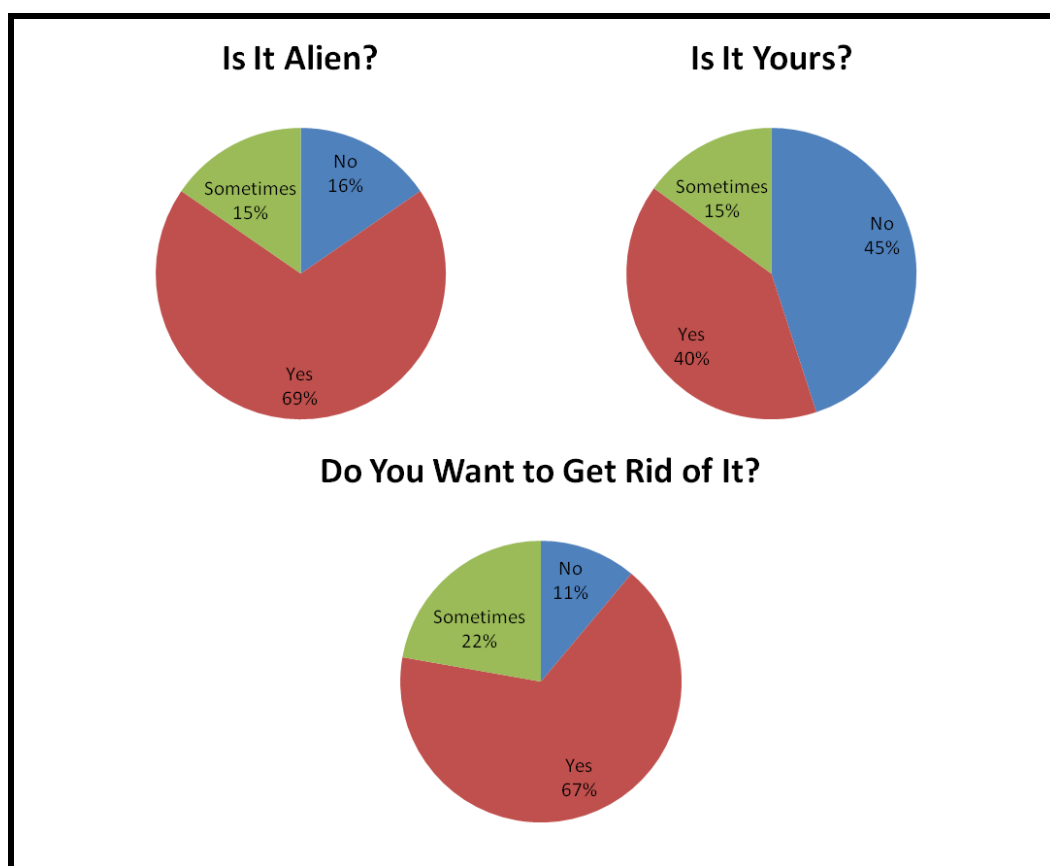
- 1) Is it yours?
- 2) Is it alien?
- 3) Do you want to get rid of it?

### 4.3.2. Results

The results are summarised below. A remarkably large proportion of CRPS sufferers experience altered perception of their affected limb, indeed 89% of those questioned would like to “get rid of it” altogether, some persistently (67%), others intermittently (22%). 45% of respondents did not consider the limb to belong to them and 69% considered it to be ‘alien’ (see table 2 and figure 9).

	Is it yours?	Is it alien?	Do you want to get rid of it?
<b>No</b>	18 (45)	6 (15)	5 (11)
<b>Yes</b>	16 (40)	27 (70)	30 (67)
<b>Sometimes</b>	6 (15)	6 (15)	10 (22)
<b>Total</b>	40 (100)	39 (100)	45 (100)

**Table 2 Results of Limb Perception Questions.** The percentage of people giving each response is given in brackets.



**Figure 9 Results of Limb Perception Questions** The majority of respondents reported some feelings of alienation or disownership towards their affected limb.

### 4.3.3. Discussion

In many ways these results are exactly as one might expect, if a part of the body is causing such pain and discomfort then to remove that part would provide some relief. What is perhaps of greater interest is that almost half of respondents did not consider the limb to be their own and over two thirds considered it 'alien' to them. Some of the comments made in response to the above questions show how strongly sufferers feel about their CRPS limb:

"It belongs to a freak"

"It doesn't feel like mine, despite the constant and extensive pain it can feel disconnected and alien. I have wanted to get rid of it repeatedly..."

"Doesn't feel like it's my limb somebody else's. Wanted to cut it off in the bath so I wouldn't make a mess."

Neglect and alien hand syndrome (AHS) patients often have similar reactions to a limb following damage to the cerebral hemisphere contra-lateral to that limb. Pain can cause an altered perception of body image in humans and as discussed below previous work has shown CRPS patients to have altered mapping of the affected limb within S1 (Forderreuther *et al.*, 2004, Maihofner *et al.*, 2003).

Impaired perception of the hand has previously been demonstrated in patients with upper limb CPRS, Forderreuther *et al* (2004) examined 114 patients and found 54.4% described their affected hand as foreign. The majority of these patients (35%) voluntarily gave up this information and the remainder selected terms suggesting foreignness from a variety of suggested words. Although 88.9% of patients with chronic CRPS (duration of more than 1 year) reported such feelings 45% of acute sufferers (duration less than 90 days) also had feelings of foreignness. This suggests that such symptoms either increase over time or are indicative of the severity and prognosis of the disease. 48% of patients were unable to identify individual fingers following

tactile stimulation on the affected side compared to only 6.5% on the unaffected side suggesting altered representation of the affected hand within the sensory cortex.

As a measure of neglect these authors used the line bisection task. Subjects are required to centrally bisect a line on a piece of paper that is placed either straight in front of them or to their left or right hand side. Deviations from the centre of the line are measured in millimetres. No significant difference was found between controls and patients with CRPS of the left limb, patients suffering with right sided CRPS demonstrated some rightward deviation when using the left hand in the centre and right condition. These results do not fully support the 'neglect-like' hypothesis since they were not seen in all patients, also classic neglect is more commonly associated with lesions of the right hemisphere and so affects the left side of the body (Farne *et al.*, 2004), the results reported here only show problems with the right hand. Also neglect patients tend to be unaware or in denial of their symptoms, the patients studied here were fully aware of their feelings towards their affected limb. Clearly there are some similarities with neglect and the symptoms described here and it is likely that altered sensorimotor processing is at the root of both problems and similar techniques may be beneficial in treating both cases.

Taken together with the results reported here it can be suggested that in CRPS changes within the central nervous system result in an altered perception of the body, perhaps due to plasticity of the cerebral cortex. Further work is necessary to identify the root cause of these changes and improve current treatment methods.

## **4.4 Referred Sensations**

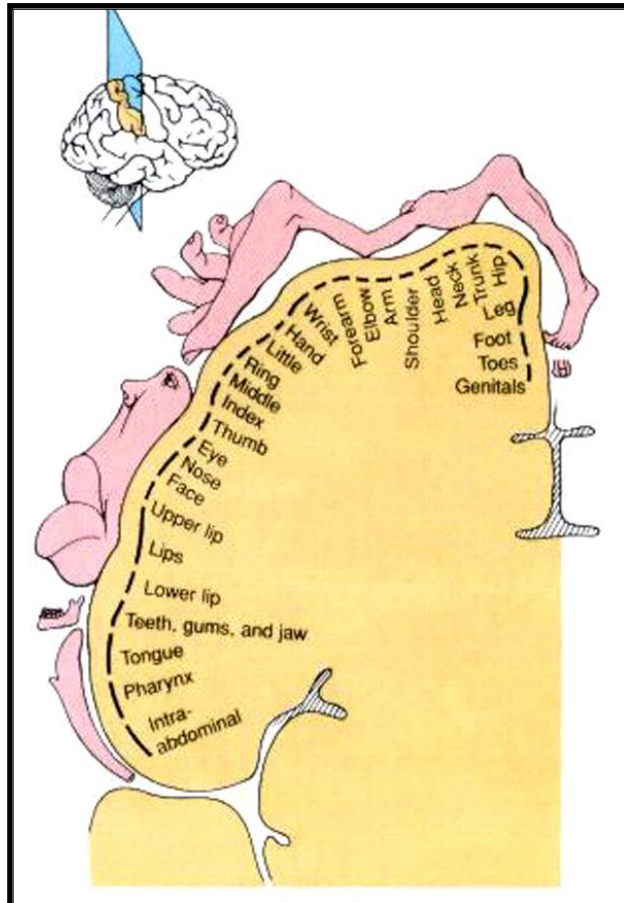
### **4.4.1 Introduction**

Referred sensations (RS) are defined as stimulation in one part of the body resulting in a sensation in another (unstimulated) area, perhaps the most

well-known occurrence of this phenomenon is phantom limb pain where sensations (often pain) are 'felt' in a limb that has been amputated. Over time these sensations tend to diminish in such a way as the limb appears to 'telescope', in the case of an amputated arm most of the arm disappears and amputees are often left with the feeling of a hand or fingers on the end of the stump (Harris, 1999, Moore *et al.*, 2000, Whyte and Niven, 2001). RS have been reported in chronic pain and specifically in CRPS (McCabe *et al.*, 2003a).

The basis of RS appears to be some form of cortical remapping, this has been demonstrated in many conditions including CRPS, repetitive strain injury and post amputation (Byl *et al.*, 1997, Flor, 2003). The sensory cortex is made up of a 'map' of the body (see Figure 10 below), with specific areas being activated when a specific part of the body is stimulated. RS appear to be the result of alterations to the organisation of this map whereby areas overlap, shrink or become 'smeared' and stimulation of one area causes activation of the area of the cortex representing another part of the body.

Alterations in S1 have been demonstrated in many imaging and magnetoencephalographic studies. In both humans and monkeys, amputation results in a shrinking of the area representing the amputated body part which is 'invaded' by adjacent cells representing other body parts. For example, following amputation of the hand or fingers, the area representing the face extends into the S1 area of the amputated hand (Flor *et al.*, 1995, Flor, 2003, Merzenich *et al.*, 1984, Weiss *et al.*, 1998). This alteration has been shown to correlate to the intensity of phantom limb pain felt by the amputee. In contrast to this, when a part of the body is used frequently for fine work its representative area in the somatosensory cortex increases, this has been demonstrated in string musicians who have a significantly increased representation of the fingers of the left hand and Braille readers whose index fingers are over represented (Halligan *et al.*, 1999).



**Figure 10 The Human S1 somatosensory cortex.** The 'Homunculus' shows the extent of representation of each body part. Regions of intense innervation such as the fingers and lips cover a much greater area than those providing less sensory input. In many conditions this map is altered resulting in blurring of the areas and misrepresentation of the stimulated body part.

The questions asked in relation to referred sensations are:

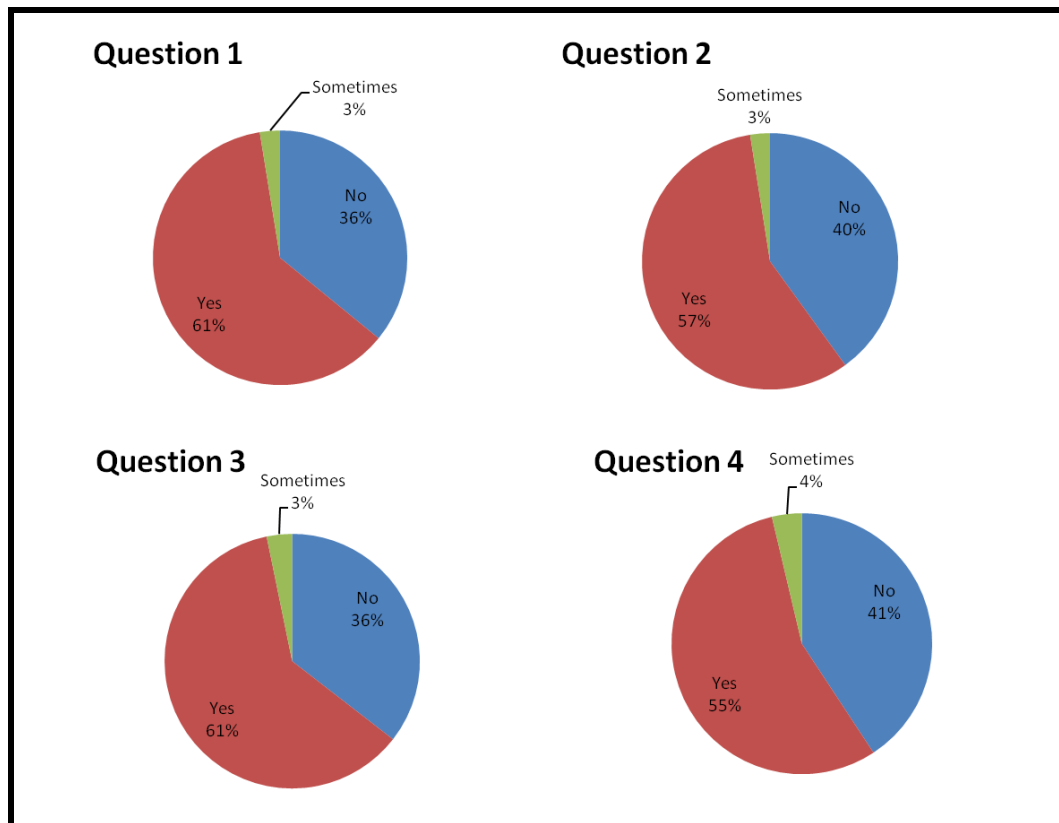
1. Does touching any part of your body cause pain in another part of your body?
2. Does touching any other part of your body cause pain in the affected area?
3. Is sensation referred between perineum and foot?
4. Is sensation referred between hand and face?

#### **4.4.2. Results**

Two thirds of patients questioned have experienced RS at some time, more than half experienced sensations hand to face and/or foot to perineum (see table 3 and figure 11).

	Question 1	Question 2	Question 3	Question 4
<b>No</b>	14 (36)	16 (40)	11 (35)	11 (41)
<b>Yes</b>	24 (61)	23 (57)	19 (62)	15 (55)
<b>Sometimes</b>	1 (3)	1 (3)	1 (3)	1 (4)
<b>Total</b>	39 (100)	40 (100)	31 (100)	30 (100)

**Table 3 Results of Referred Sensation Questions.** The percentage of people giving each response is given in brackets.



**Figure 11 Results of Referred Sensation Questions.** The majority of respondents reported referred sensations both directly involving the effected limb and in other parts of the body.

#### 4.4.3. Discussion

The results reported here support previous work demonstrating a degree of reorganisation of the cerebral cortex in CRPS. Along with RS this reorganisation may also play a role in the development of other unusual symptoms such as altered limb perception and changes in spatial awareness. In the present study two thirds of patients reported experience of RS compared with only one third in previous work (Maihofner *et al.*, 2006, McCabe *et al.*, 2003a). There are several possible reasons for this but the

most likely is that the questions here were of a more general nature whereas the two previous studies were looking at specific locations on the body. False positives are also more likely in this study as the questions due to the environment in which the questions were answered and the reliance on the memory of the subjects, it therefore seems likely that the real figure is somewhere between one and two thirds.

Both acute and chronic pain have been demonstrated to alter cortical organisation. In chronic back pain patients magnetic fields in the cerebral cortex contralateral to the site of painful stimulation were increased in response to painful stimulation compared to healthy controls (Flor *et al.*, 1997). There was also a significant increase in the distance between the area representing the index finger and that representing the back in these patients. These differences were positively correlated with chronicity (Flor *et al.*, 1995, Flor, 2003). Acute pain has been demonstrated to generate RS in healthy subjects Knecht *et al* (1998) reported sensations referred from lip to hand during innocuous stimulation following a period of painful stimulation to the finger. This coupled with evidence of RS just ten days after finger amputation in one patient (Weiss *et al.*, 1998) indicates that cortical changes may occur rapidly and evolve over time to become more permanent.

Cortical reorganisation has also been demonstrated in CRPS, Maihofner *et al* (2003) found a significant shift in the representation of the CPRS affected hand to the cortical representation of the lip as measured by MRI. This affect was positively correlated to the extent of spontaneous pain and hyperalgesia experienced by the subjects. This is supported by the work of McCabe *et al* (McCabe *et al.*, 2003a) who demonstrated RS felt in the area of the body adjacent to the stimulated area in S1 in CRPS patients. Subjects with chronic CRPS (> 1year) demonstrated much stronger RS which were not attenuated with treatment, acute sufferers (< 1 year) had much weaker RS which recovered with treatment indicating a role for chronic pain in the development of RS.



Taken together these results suggest that RS and related symptoms increase with time and may be correlated with increased pain in CRPS and other chronic pain conditions. As well as providing further evidence for pathology of the central nervous system in the development and progression of CRPS, this work also demonstrates the need to consider symptoms such as RS in diagnosis and treatment. Many of the patients questioned here had had inappropriate investigations into the origin of these sensations, for example pain in the face had led to a dental opinion and that in the perineum a urological consultation; such consultations are time consuming and may cause unnecessary worry for the patient. Further work is necessary to identify the underlying mechanisms of these symptoms and develop better methods of treatment.

#### **4.5. Chapter Discussion**

Care must be taken when interpreting these results and drawing conclusions from them since the subject group all being CRPS patients at a conference relating to the condition may be considered somewhat biased. It is however possible to draw some conclusions from these results and they certainly warrant further investigation.

Cortical remapping is a significant issue in chronic pain conditions and may be the cause of many distressing symptoms such as those described here. However, Forderreuther *et al* (2004) reported no significant link between feeling of foreignness, pain intensity and ability to identify a specific finger suggesting that they may not all be the direct result of remapping. It is likely that these symptoms are the result of alterations in many parts of the brain and that remapping is one major part of the process.

These results suggest that plastic changes in the CNS are in some way involved in the development and maintenance of chronic pain and that this can lead to alterations in processing of information within the brain. Whether these changes are causative factors or symptoms of the condition is not clear. However many neuromodulatory factors such as cytokines, chemokines and steroid hormones are known to increase in response to

chronic pain. Cytokines have been demonstrated to alter gene expression in neurons and related cells (Ledeboer *et al.*, 2005, Marsh *et al.*, 1993) and can change their sensitivity and responsiveness. Steroid hormones are known to have both stimulatory and inhibitory effects on synaptic processing in the hippocampus (McEwen, 2001, Yankova *et al.*, 2001) and have also been demonstrated to affect cell growth and differentiation in this area (McEwen, 2001, McKittrick *et al.*, 2000, Yankova *et al.*, 2001), an over reactive HPA axis or a decrease in oestrogen following the menopause may therefore contribute to cortical changes.

## **Chapter 5**

# **5 Experimental: Development of a Rat Inflammatory Pain Model.**

### **5.1 Introduction**

The following experiments were carried out in accordance with UK legislation, Animals (Scientific Procedures) Act, 1986 Project Licence: PPL 30/2195 and Personal Licence: PIL 30/7926. Supervision was provided by the project licence holder, Dr Vivienne Winrow and assistance with animal husbandry and euthanasia received from the technical staff of the Biosciences Services Unit at the University of Bath.

Although there is growing evidence for plastic changes within the CNS as a result of neuropathic pain the effect of persistent peripheral inflammatory pain on the CNS has not been examined in great detail to date and existing results vary. In order to investigate the effects of peripheral inflammatory pain on the brain a model is required that will produce persistent (> 2 weeks) inflammatory pain that is confined to a specific region. Two inflammatory models were tested both involving injection of an inflammogen into one hind paw of the rat, the inflammogens tested were: a) 1% Carrageenan and b) 0.01%, 0.1% and 1% CFA (see Chapter 3 for further information). These models were compared in order to find the optimum inflammogen and concentration that would provide a persistent painful response with minimum systemic involvement and distress to the animals.

## **5.2 Materials and Methods**

### **5.2.1. Materials**

#### **5.2.1.1 Animals**

Adult Male Wistar rats with initial weights of 200-230g were used in all experiments (Charles River UK Ltd., Margate, UK).

#### **5.2.1.2 Reagents**

Incomplete Freund's adjuvant, Carrageenan, and sterile physiological saline (0.9% w/v) (Sigma-Aldrich CO Ltd., Poole, UK). *Mycobacterium tuberculosis*, freeze dried human strains C, DT and PN mixed (Central Veterinary Laboratories, Surrey, UK). Isoflurane, Oxygen and Liquid Nitrogen (BOC, Surrey, UK). Isopentane (Fisher Scientific, Leics, UK). Hypoxyprobe (HPI Inc., Burlington, MA, USA).

#### **5.2.1.3 Equipment and Consumables**

Sterile needles and syringes (Becton Dickinson, UK).

Pestle and Mortar (Mason & Cook, Stoke-on-Trent, UK).

Plethysmometer (Ugo Basile, Comerio, Italy).

### **5.2.2 Animal Model**

All experiments were carried out under Home Office licence, project number PPL 30/2195 and personal licence number PIL 30/7926.

#### **5.2.2.1 Animal Husbandry**

Rats were housed in groups of 3 or 4 to a cage and exposed to a 12 hour light/dark cycle and an average temperature of  $20 \pm 2^{\circ}\text{C}$ . Sawdust was used as bedding and each cage contained a plastic tube to provide environmental enrichment for the animals. Animals were given access to tap water and

standard rat diet *ad libitum*. Animals were weighed regularly as part of monitoring their general health.

On arrival in the facility rats were separated into groups of four and given a minimum of five days to rest and acclimatise to their surroundings prior to commencement of the experiment. The experimenter was blinded to the grouping procedure.

#### **5.2.2.2 Induction of Footpad Inflammation**

Animals were lightly anaesthetised by inhalation of 3-4% isoflurane in oxygen and given an intraplantar injection of 100µl of 0.01%, 0.1%, 1% CFA in 1:1 saline emulsion, 1% carrageenan or physiological saline into the right hind-paw. Non-injected controls were also anaesthetised. Animals were allowed to recover under supervision.

#### **5.2.2.3 Measurement of Paw Volume**

The volume of both hind paws was measured by liquid displacement plethysmometry using the Ugo Basile plethysmometer (see figure 12). This device consists of two chambers containing electrodes and filled with saline solution, the animal is gently restrained and the paw lowered into the larger chamber, saline of equivalent volume to the paw is displaced into the second chamber and the subsequent change in conductance measured and converted into a volume measurement on the electronic display. In order to ensure consistency measurements were all carried out by the same experimenter and paws were immersed up to the ankle joint until the calcaneum was fully submerged.

#### **5.2.2.4 Termination and Tissue Harvest**

One hour prior to termination animals were lightly anaesthetised with 3-4% isoflurane in oxygen and 60 mg/kg Hypoxyprobe was administered via intraperitoneal injection. At the end of the experiment, animals were killed by cervical dislocation and immediately decapitated using a guillotine. Brains

were rapidly removed and frozen in freezing/melting isopentane over liquid nitrogen. Frozen brains were kept in liquid nitrogen for transportation prior to longer term storage at -70°C.



**Figure 12 The Ugo Basile Plethysmometer.** The rat paw is placed into the larger saline containing chamber and the difference in conductance between it and the smaller chamber is measured and converted into volume. *Image Ugo Basile, Italy.*

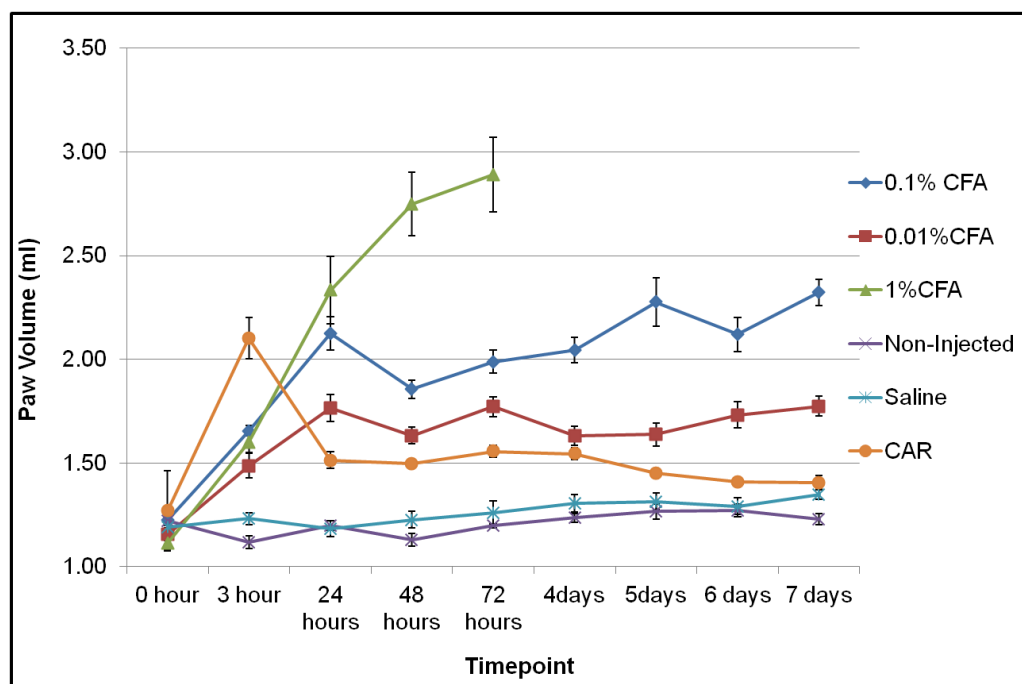
#### **5.2.2.5 Statistical Analysis**

Treatment groups were compared using analysis of variance (ANOVA) followed by Tukey's honestly significant difference test (Tukey's HSD) for post-hoc analysis. Within group analysis to compare left and right feet was performed using a paired sample t-test. All analyses were performed using SPSS and  $p < 0.05$  was considered significant. Graphs display mean values and error bars represent  $\pm$  the standard error of the mean (SE).

## 5.3 Results

### 5.3.1 Paw Volume Measurements from Pilot Experiments

#### 5.3.1.1 Comparison of paw volume in CAR and CFA models



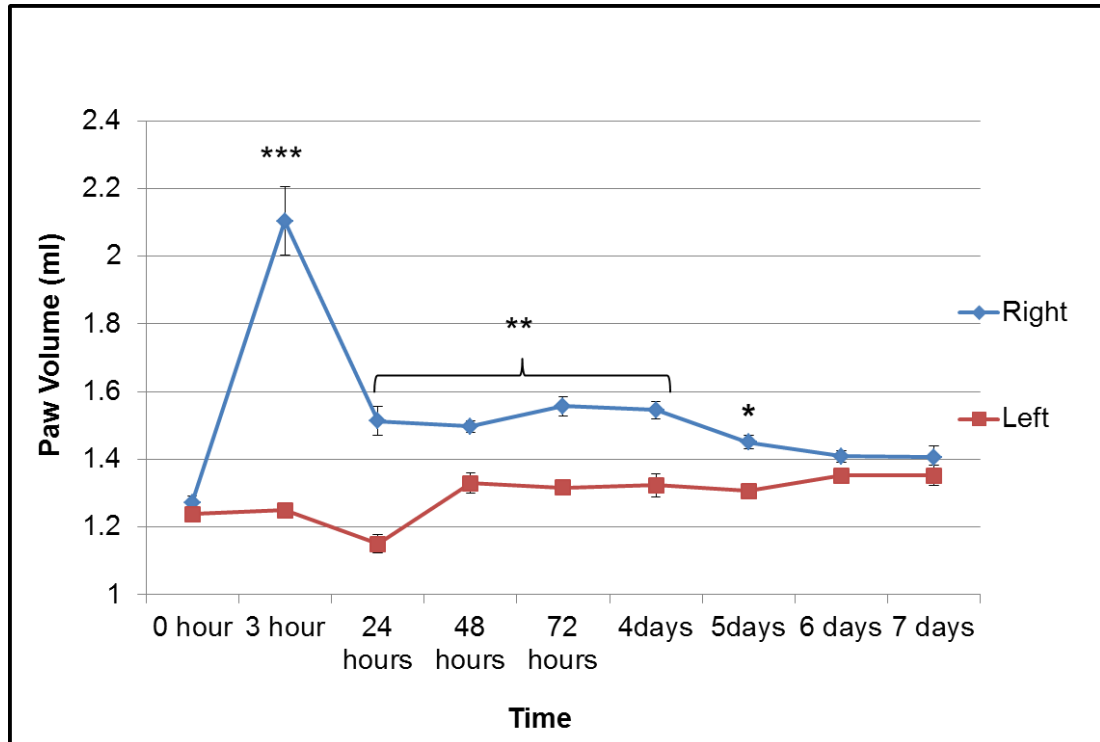
**Figure 13 Right (injected) hind paw volumes comparing treatments and controls.** CAR injected paw volume peaks at 3 hours and then declines to 7 days where it returns to normal. 1% CFA injected paws rapidly swelled until 72 hours at which point the experiment was terminated due to severe discomfort of the animals. 0.1% injected paw volume peaked at 24 hours dropping to 48 and then steadily rising, 0.01% volumes did not show such a significant peak and were similar to those of CAR, as such 0.1% CFA was chosen for experimentation. There were no significant differences between saline and non-injected controls. For further information see text below, n=8 for all groups other than CAR and saline where n=6.

#### Carrageenan

CAR injection resulted in rapid swelling of the injected hind paw which peaked at three hours post injection and then proceeded to decrease back to baseline. At the three hour time point CAR injected paws were significantly larger than all other groups ( $p < 0.05$ ), from 24 hours onwards their volume was less than that of other treatment groups and by five days there was no longer a significant difference compared with either of the two controls.

When injected paws were compared with non-injected left hind paws in the same group similar results were found as compared with right hind paws of

control animals. There was a highly significant difference in paw volume from days 3-5 ( $p<0.01$ ) which decreased by day 5 ( $p<0.05$ ) and was no longer significant at days 6 and 7 (see figure 14).



**Figure 14 Hind paw volumes of 1% CAR injected animals.** The volume of the right (injected) paws increased rapidly, peaking at 3 hours where the difference from left paws was highly significant. Right paw volume then decreased but remained significant from 24 hours to four days) and five days returning to baseline at 6 and 7 days. \*\*\*  $p<0.001$  \*\*  $p<0.01$  \*  $p<0.05$   $n=6$

#### Complete Freund's Adjuvant

0.01% CFA resulted in a gradual increase in volume of the injected paw with minor peaks at 24 and 72 hours. This increase was significant versus both controls from 3 hours onwards ( $p<0.01$ ) and also versus CAR at both days 6 and 7 (see figure 13).

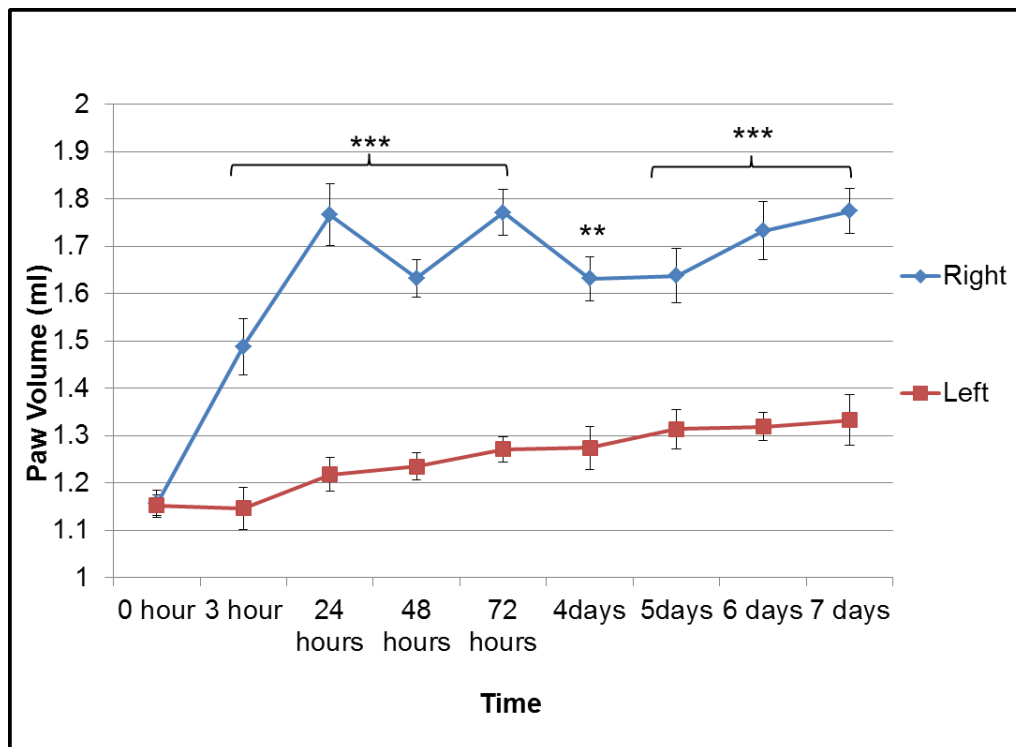
There was a more rapid increase in right paw volume in animals injected with 0.1% CFA leading to a more pronounced peak at 24 hours. Volume then increased steadily from 48 hours with a minor peak at 5 days. From three hours onwards, injected paw volume was significantly greater than that of both control groups ( $p<0.05$ ), differences were also significant when



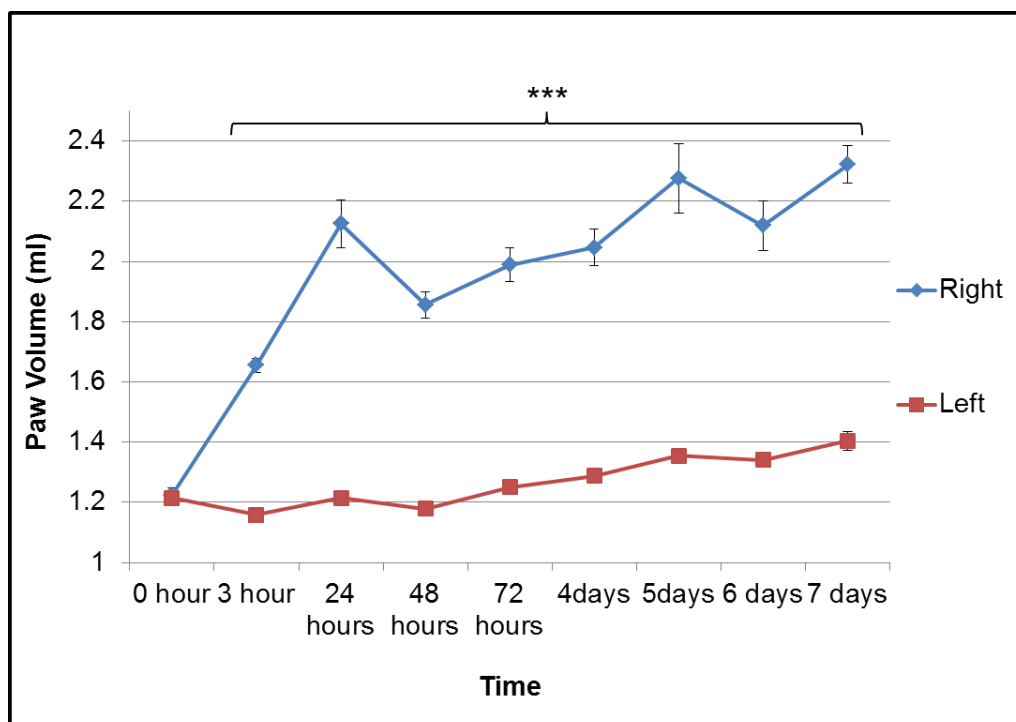
compared with CAR injected paws from 24 hours and 0.01%CFA injected paws from 4 days, (see figure 13).

Animals injected with 1% CFA had the highest increase in right paw volume, unfortunately this resulted in significant distress to the animals by day 3 and it was decided to terminate the use of this concentration at that time point. Intravenous injection of CFA is known to induce systemic arthritis and these animals showed early symptoms of this disease. As with other concentrations of CFA, right paw volume was significantly increased compared with both control groups from the three hour time point. At 24 hours the difference was significant between 1% and CAR or 0.01%CFA and at 48 and 72 hours the 1%CFA injected animals had significantly larger right paws than all other groups. As mentioned above there was also a significant difference between right paw volume of this group and the CAR injected animals at baseline, (see figure 13).

When comparing right (treated) and left hind paws (untreated) in the same animals the same pattern of significance as when compared with controls was seen in both 0.01% and 0.1% CFA groups with the right paw being significantly larger than the left from 3 hours to 7 days ( $p < 0.01$ ) (see figures 15 and 16).



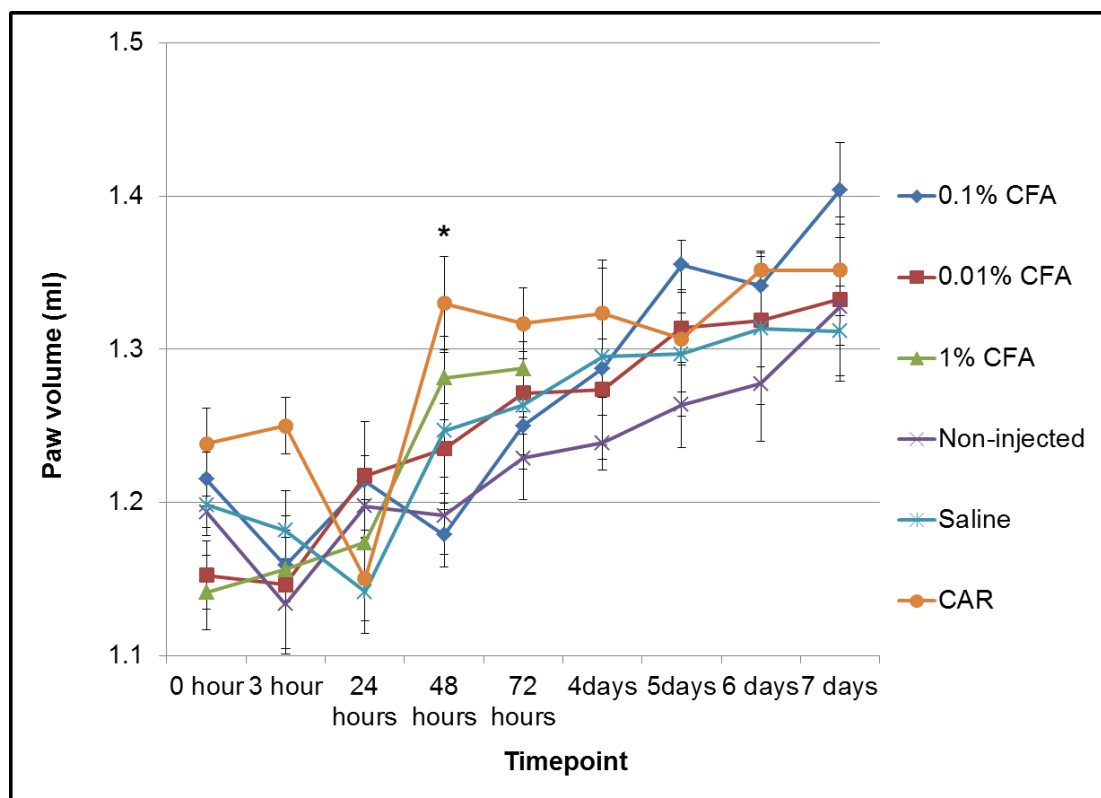
**Figure 15 Hind paw volumes of 0.01%CFA injected animals.** A gradual increase in right (injected) paw volume was seen with minor peaks at 24 and 72 hours, the difference between paws was significant at all time points post injection. \*\*\*  $p < 0.001$  \*\*  $p < 0.01$   $n = 8$ .



**Figure 16 Hind paw volumes of 0.1%CFA injected animals.** Right paw volume gradually increases post-injection peaking initially at 24 hours and then dropping slightly prior to increasing gradually up to 7 days the difference between right (injected) and left (non-injected) paws was highly significant at all post-injection time points. \*\*\*  $p < 0.001$   $n = 8$ .

## Non-injected Paws

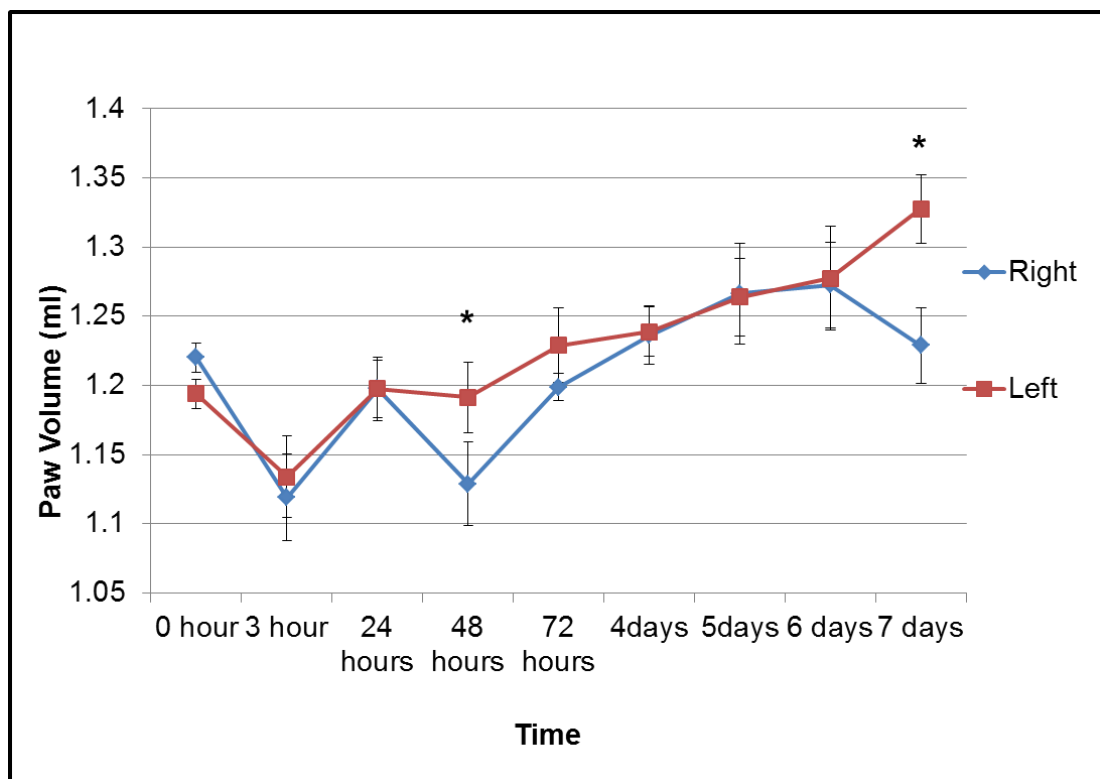
There were no significant differences in left paw volumes between treatment groups and controls other than between CAR treated animals and non-injected controls at the 48 hour time point ( $p < 0.05$ ). A peak was also seen in left paw volume of CAR treated animals at the 3 hour time point although this was not significant, this result is interesting as a similar result with concomitant cellular inflammation has previously been reported (Roberts *et al.*, 2006). The lack of “mirror-image” inflammation seen in the CFA treated animals and at the later time points in CAR animals may be explained by the differences in the early and late inflammatory responses. Other than this there were no significant differences in left paw volumes, a slight increase over the 7 day period is apparent as would be expected with normal growth of the animals, (see figure 17).



**Figure 17 Left (non-injected) hind paw volumes comparing treatments and controls.** There was a significant difference between the left paws of CAR animals when compared with non-injected controls at the 48 hour time point \*  $p < 0.05$   $n=8$  for all groups other than CAR and saline where  $n=6$ .

### Control Groups

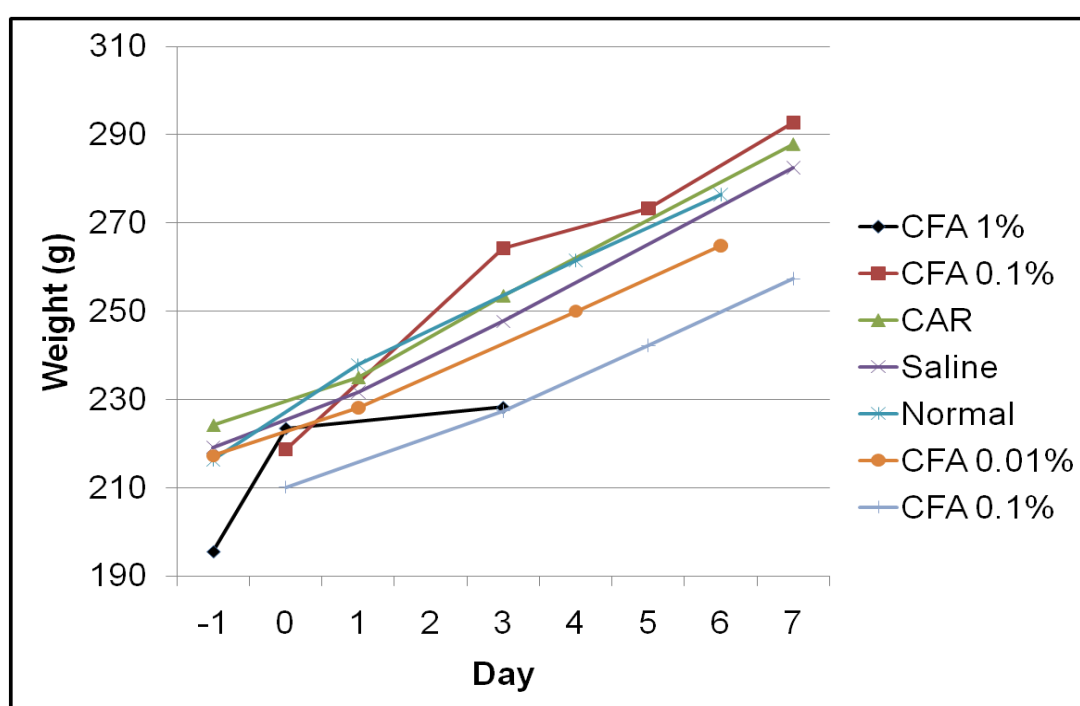
Although there are some minor variations in the size of the right paws of saline injected and non-injected animals there was no significant difference between these two groups at any time point. When comparing left and right paws within groups of control animals there were no-significant differences in paw volume within the saline group (data not shown). There were no significant differences in left and right paw volumes of non-injected controls, (see figure 18). There was a significant difference in the volume of left and right paws at 48 hours and 7 day time points.



**Figure 18 Hind paw volumes of non-injected controls.** The left paws were significantly larger than the right in non-injected controls at 48 and 7 day time points. \* $p < 0.05$   $n = 4$ .

### 5.3.1.2 Comparison of weight change in CFA and CAR models

In all models, other than 1%CFA, animals gained weight at approximately the same rate suggesting no systemic effects of the treatment. In the 1%CFA treated group there is no apparent weight increase between days 1 and 3 suggesting there is some systemic involvement in these animals, (see figure 19). Weight loss is a symptom of systemic arthritis and, as discussed above it is likely that animals treated with 1% CFA were displaying early signs of this disease.



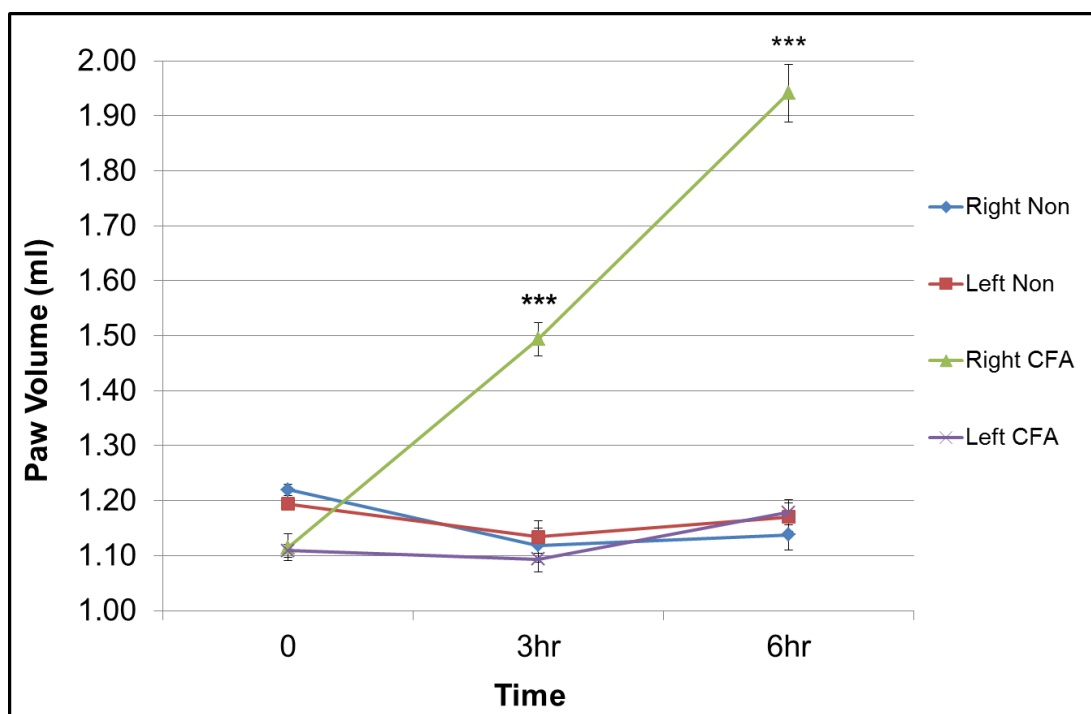
**Figure 19 Effects of treatment on weight gain.** In general weights increased at a steady rate over time. 1% CFA injected animals did not gain weight at the same rate, however, suggesting some systemic involvement in this group.

### 5.3.2 Paw Volume Measurements in Complete Freund's Adjuvant Model

Following the pilot experiments it was decided to use 0.1% CFA as the inflammatory stimulus over several time points up to fourteen days. Eight animals were used in each group and compared with non-injected controls. The results for each time point are presented here and will be discussed in further detail in the later experimental chapters. The time points were chosen to represent different stages of the development of persistent pain, 6 hours representing the acute stage, 24 and 72 hours the sub-acute stage and 7 and 14 days the beginning of a persistent pain state.

#### 5.3.2.1 Six Hour Time point

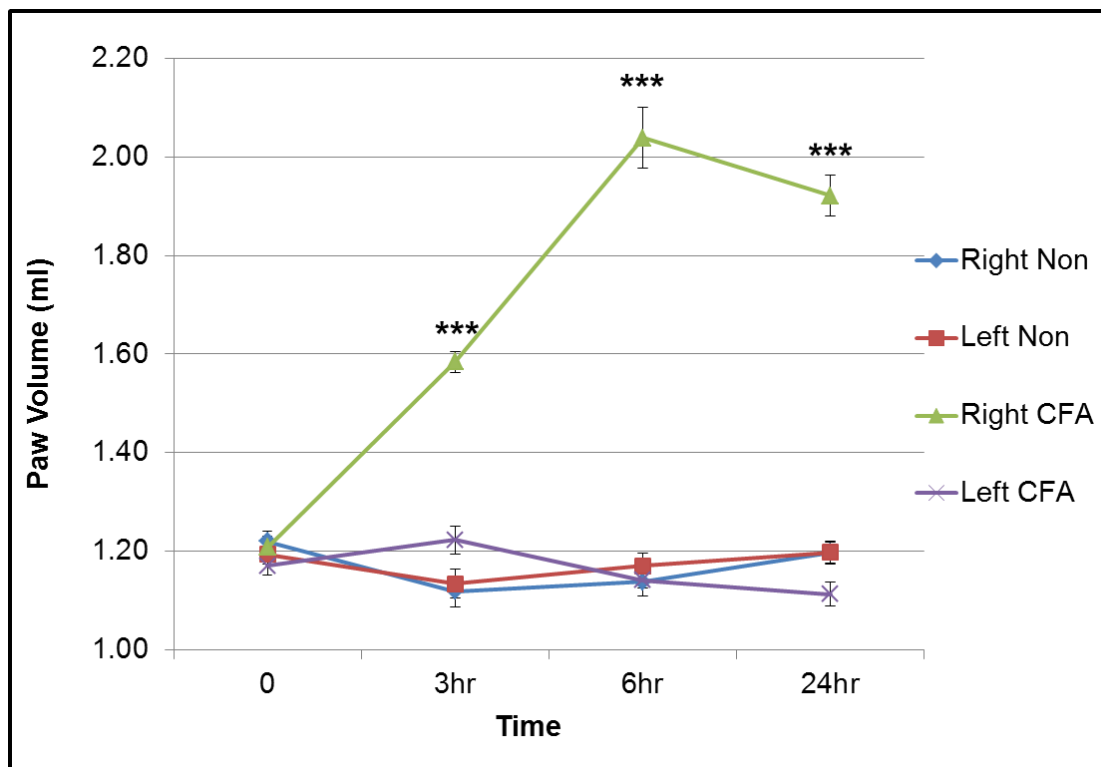
The right hind paws of the injected animals were significantly larger than either left or right hind paws of non-injected animals and the non-injected left paws of the treatment group at three and six hours post injection ( $p < 0.001$ ). (see figure 20).



**Figure 20 Paw Volumes Non-Injected Control and 0.1% CFA 6 hour time point.** The volume of the CFA injected paw increased rapidly over six hours and was significantly larger than the contralateral non-injected paw and the paws of the control group at both three and six hours post injection  $n=8$  \*\*\*  $p < 0.001$ .

### 5.3.2.2 24 Hour Time point

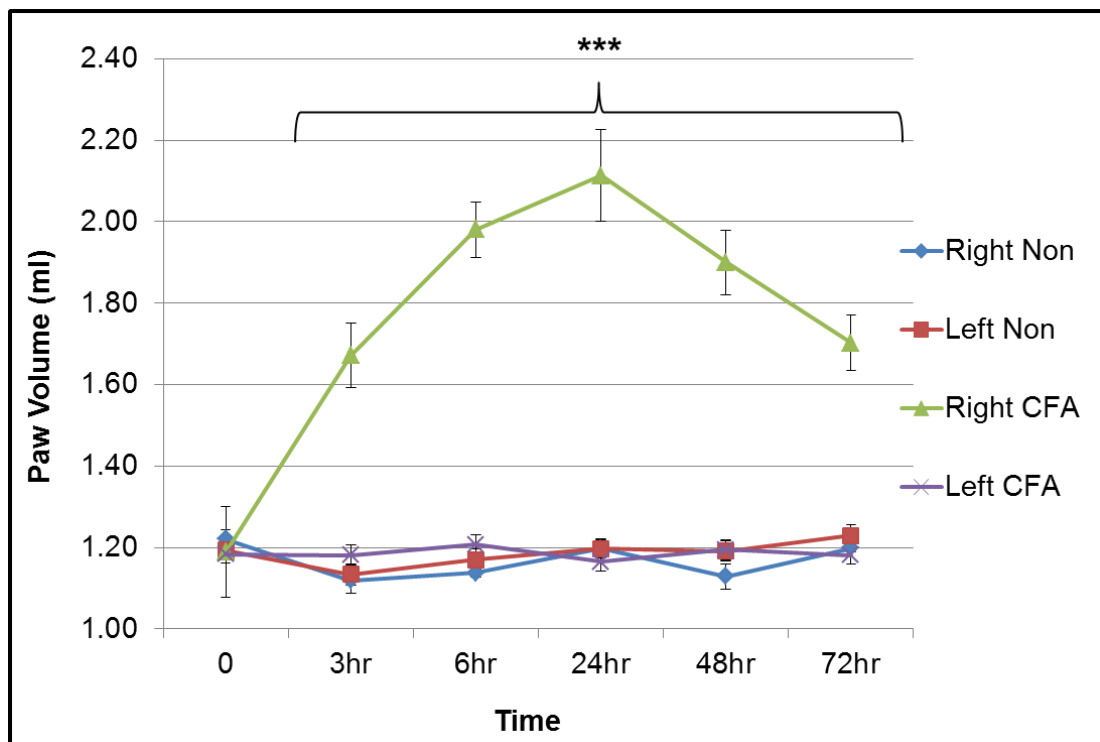
There were no significant differences in paw volume at the start of the experiment. There was a rapid increase in injected paw volume and at 3, 6, and 24 hours post injection the right paws of the CFA injected group were significantly larger than the non-injected contralateral paws and both paws in the control group ( $p<0.001$ ) (see figure 21).



**Figure 21 Rat paw volumes 0.1% CFA versus non-injected controls 24 hour time point.** Injected paw volume decreases slightly to 24 hours following a relatively rapid increase in the first 6 hours post-injection, the difference in volume is still highly significant at this time point when compared with the contralateral paw and non-injected control animals.  $n=8$  \*\*\*  $p<0.001$ .

### 5.3.2.3 72 Hour Time point

Following the increase in swelling of the injected paw as described above there is a peak at 24 hours after which swelling decreases to 72 hours. The injected hind paw of CFA animals was significantly larger than the contralateral non-injected paw and both paws of animals in the control group at all time points post-injection ( $p < 0.001$ ) (see figure 22).

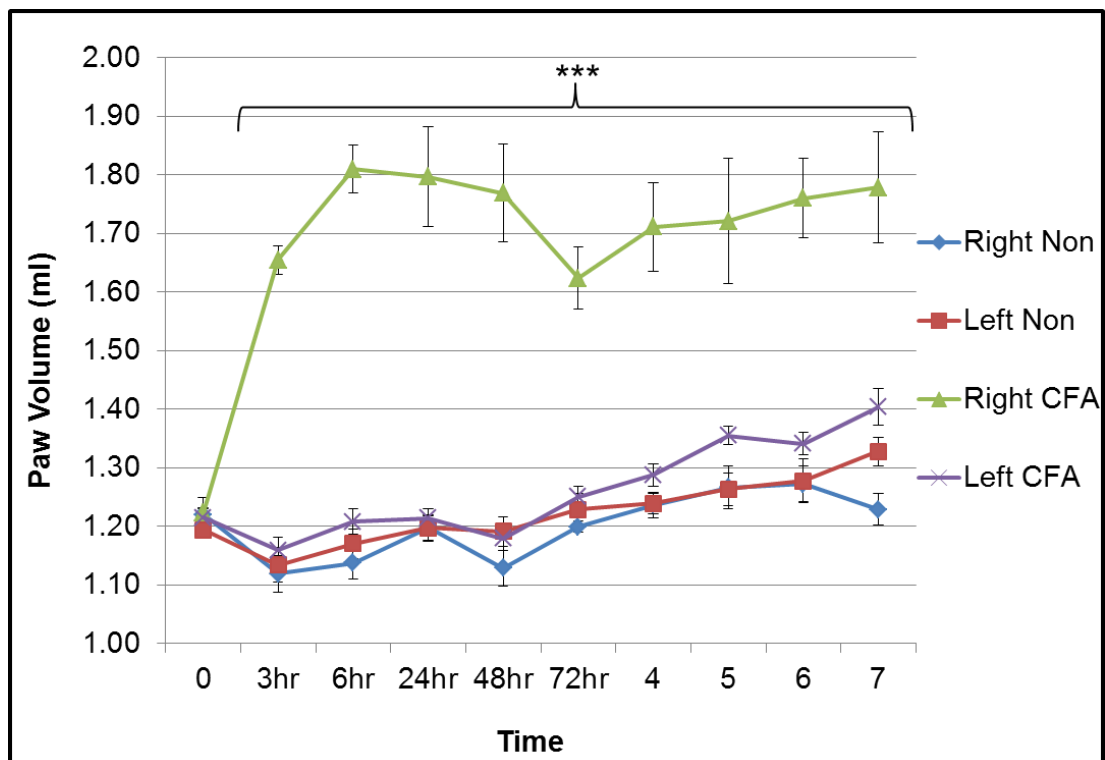


**Figure 22 Rat paw volume 0.1% CFA versus non-injected controls 72 hour time point.** Following the peak at 6-24 hours volume of the injected paw decreases to 72 hours, the difference between injected paws and contralateral paws and non-injected control animals is highly significant at all post-injection time points.  $n=8$  \*\*\*  $p < 0.001$



#### 5.3.2.4 7 Day Time point

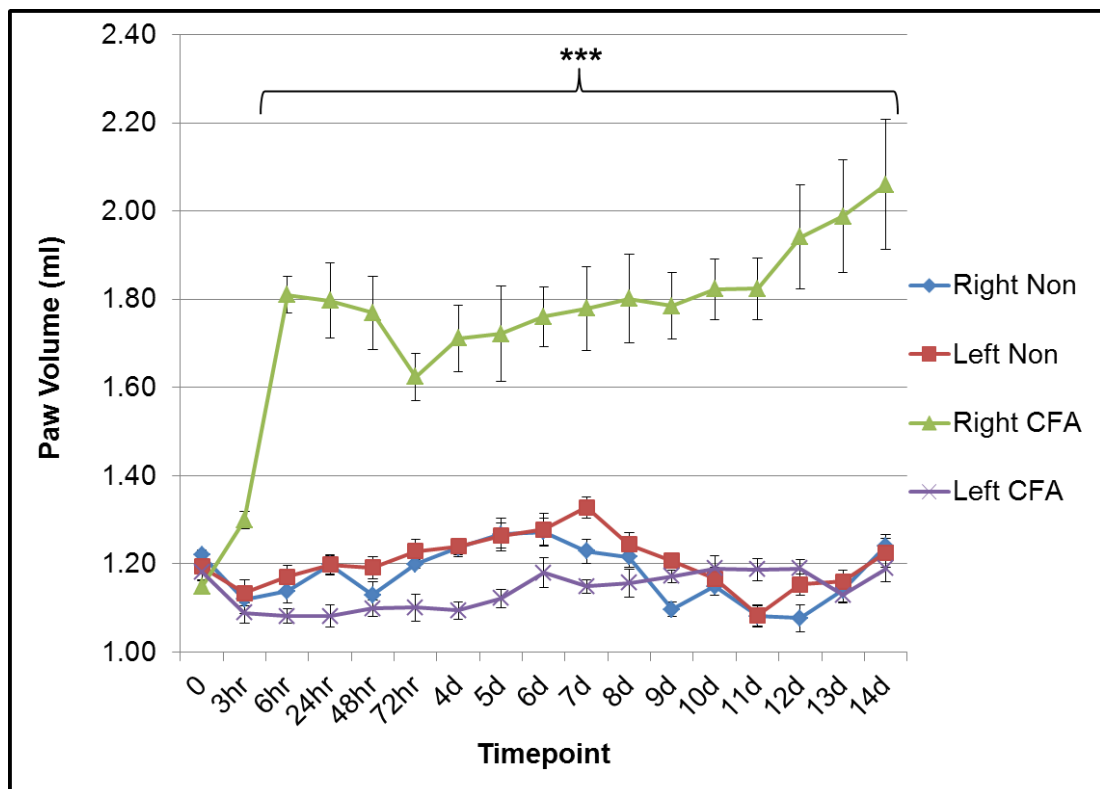
Following a peak at 6-24 hours and a decrease in swelling up until the 72 hour time point the injected paw steadily increases in volume until the 7 day time point. Injected paws were significantly larger than non-injected contralateral paws and both paws of control animals at all post injection time points ( $p<0.001$ ) (see figure 23).



**Figure 23 Rat paw volume 0.1% CFA injected vs. non-injected controls over 7 days.** Following a peak in swelling at 6-24 hours the injected paw decreased in volume until 72 hours after which there is a steady increase in volume to 7 days. Injected paws were significantly larger than contralateral and control non-injected paws at all post-injection time points.  $n=8$  \*\*\*  $p<0.001$ .

### 5.3.2.5 14 Days

Looking at the 14 day time point the overall pattern of paw swelling from the acute to the persistent stage can be observed. Following injection there is a rapid increase in volume which peaks at 6-24 hours, there is then a decrease to 72 hours from which point volume increases gradually until at least 14 days post-injection (see figure 24).



**Figure 24 Rat paw volume 0.1% CFA injected vs. non-injected controls over 14 days.** The 14 day paw volume profile shows the change in swelling seen in the injected paw over the entire experiment, The injected right hind-paw swells fairly rapidly over the first 24 hours, peaking at 6-24 hours, the swelling there is a dip in volume to 72 hours followed by a gradual increase to at least 14 days. n= 8 \*\*\*p<0.001.

## 5.4 Discussion

The initial aim of this chapter was to identify a suitable animal model for use in experiments investigating persistent pain. Consistent with previously published results in the CAR model the injected paw volume peaked at 3 hours and rapidly returned towards baseline in a manner characteristic of an

acute inflammatory response. This inflammation is regulated by the immediate release of inflammatory mediators such as bradykinin, histamine, tachykinins and complement which are released both from local cells and infiltrating cells such as neutrophils and subsequently quelled by phagocytosis of neutrophils by monocytes (Morris, 2003, Lima and Almeida, 2002). The significant difference between the CAR injected paw and both the contralateral and control group paws decreased towards baseline after the 3 hour peak and was no longer significant at 6 and 7 days post-injection (see Figure 14 and ). Since the time course and persistence of pain, as measured by allodynia and hyperalgesia, have previously been demonstrated to follow a similar time course to the oedema measured here it was decided that CAR injection was not a suitable pain model to investigate chronic pain mechanisms (Stein *et al.*, 1988, Raghavendra *et al.*, 2004).

The injection of CFA produced a more persistent response than CAR at all concentrations. At the highest concentration of 1% CFA the experiment had to be terminated at 72 hours due to the obvious discomfort of the animals which, when accompanied by a decrease in weight gain, was indicative that the CFA was having a systemic effect possibly due to the early stages of systemic arthritis.

Both 0.01% and 0.1% CFA injected paws showed a significant increase in volume when compared with contralateral and control group non-injected paws and this swelling persisted for at least 7 days post injection (see figures 15 and 16). A specific profile of swelling was more apparent in the 0.1% injected animals, an initial peak was evident at 24 hours and swelling then decreased to 72 hours before gradually increasing until at least 7 days. It is likely that the initial peak is the result of an acute inflammatory response to a foreign substance, as seen in the CAR model, which rapidly subsides resulting in a minor decrease in paw volume at 72 hours. From 72 hours post-injection swelling increases up until at least 7 days as a result of the cell mediated immune response which has been shown to continue for up to one month (Stein *et al.*, 1988, Raghavendra *et al.*, 2004, Nagakura *et al.*, 2003). Previous work has also shown that the increased swelling seen in the CFA

model is paralleled by an increase in pain, as measured by allodynia and hyperalgesia (Nagakura *et al.*, 2003, Stein *et al.*, 1988, Raghavendra *et al.*, 2004). Furthermore, Nagakura *et al* (2003) also found a significant increase in thermal hyperalgesia 24 hours post-injection which decreased at 72 hours post-injection before very gradually increasing toward 14 days. Interestingly, in this model, mechanical allodynia peaked at 7 days post-injection and continued to increase relatively rapidly to 14 days. It is possible, therefore, that thermal hyperalgesia is mediated primarily by the initial acute inflammatory response and that mechanical allodynia occurs as a result of the slower but more persistent immune response.

As a result of the pilot experiments in section 5.3.1 it was decided to use 0.1% CFA as the inflammogen in all future experiments since this concentration gave the best representation of the various stages of the inflammatory response without any apparent systemic involvement or excessive stress to the animals. The 0.01% CFA treatment was also discounted as at the latter time points the volume of the injected paw was not significantly different to that of CAR injected paws suggesting that inflammation and associated pain may not persist much further than 7 days. As no significant difference was seen between saline and non-injected control paw volumes it was decided to use one group of non-injected control animals throughout the 14 day test period.

## Chapter 6

# 6 Experimental: Gene expression of inflammatory mediators within the brain in peripheral inflammation.

## 6.1 Introduction and Aims

### 6.1.1 Introduction

The relationship between pain and inflammation in the periphery is well documented, the release of inflammatory mediators in response to tissue damage or infection leads to sensitisation of local nerve fibres and vasodilation resulting in oedema and flare (Hellstrom *et al.*, 2005, Hoheisel *et al.*, 2005). Over recent years the CNS, previously considered to be immune-privileged, has been found to have its own immune system, which is comprised of the astrocytes and microglia. When activated these glial cells release a range of inflammatory mediators including cytokines and chemokines (McMahon *et al.*, 2005). Much work within the pain research field has recently focussed on the role of glial cells and the mediators they release in the initiation and maintenance of pain states, particularly within the spinal cord (DeLeo and Yezierski, 2001, Watkins *et al.*, 2001, Bee and Dickenson, 2007). Although earlier work focussed on the role of the spinal cord there is increasing evidence that immune responses in the brain may also contribute to the development and persistence of pain, changes in the brain are also of significant interest as they likely contribute to the affective-motivational symptoms of pain which are often intractable.

Apkarian *et al* (2006) investigated the expression of IL-1 $\beta$  in rats with either CCI or SNL induced neuropathic pain. They found that SNL resulted in a significant increase in IL-1 $\beta$  expression in the brainstem at 10 days returning to baseline 24 days post-injury, within the thalamus, however, a significant increase was not seen until 24 days post-injury and was only evident on the

right side of the brain, contralateral to the injured nerve. Within the prefrontal cortex a significant increase in IL-1 $\beta$  expression was again seen at 10 days post-injury again only on the contralateral side of the brain. There was no significant difference in IL-1 $\beta$  expression in the CCI model other than then a significant decrease within the right side of the thalamus at 10 days if both CCI and SNL data were combined, however there was a trend towards an increase as seen in the SNL model. These data clearly show not only that peripheral injury causes significant changes in the brain, but also that different models, even when both represent a neuropathic injury, can produce very different results.

In a CCI mouse model it has been shown that there are rapid changes in cytokine mRNA expression over the first few hours following injury (Uceyler *et al.*, 2008), within the spinal cord TNF $\alpha$  expression decreased significantly at 6-9 hours post-injury returning to baseline at 12 hours, in the hypothalamus however, there was a drop in expression from 3-24 hours reaching a significant minimum at 24 hours post-injection before returning to baseline. Within the hippocampus and thalamus levels remained low from 6-72 hours and 1-24 hours respectively apart from minor increases in both areas at 9 hours. In the same model IL-1 $\beta$  mRNA expression was show to be decreased in the hippocampus at 1 hour and 24 hours post-injury while in the thalamus there was an increase in expression at 3 hours. The only change seen in the spinal cord was a decrease in expression 3 hours post-injection. Interestingly these results are contrary to what is commonly seen in the periphery and the CNS following a neuropathic injury since they primarily show a decrease in cytokine expression, this may be because this study concentrates on early time points and very specific brain areas as opposed to the whole brain. Furthermore the use of mice in this work might also explain the differences as most published work is from rat models.

The majority of work investigating the expression of inflammatory mediators in the CNS in pain states has focussed on neuropathic models, Raghavendra *et al* (2004) used the CFA footpad model to build on this work with an inflammatory model. They found an increase in mRNA and protein of IL-1 $\beta$ ,

IL-6 and TNF $\alpha$  in the brains of CFA injected rats at 4 hour, 4 days and two weeks post-injection. There was an increase in the glial marker, GFAP, at 4 and 14 days post-injection. Hyperalgesia and allodynia were present in the injected foot at all time points and, interestingly, thermal hyperalgesia developed in the left foot at 14 days post-injection. These authors suggest that the hyperalgesia is a result of glial activation in the brain perhaps due to glial cross talk from the left (contralateral to injected paw) to the right (ipsilateral) hemispheres, however since no comparisons were made between left and right brain regions this can only be speculative.

The chemokines are small inflammatory molecules that derive their name from their activity as chemotactic cytokines, they are largely promiscuous ligands which act on multiple receptors. The chemokine fractalkine, also known as CX3CL1 and neurotactin, binds to only one receptor, CX3CR1, and as such is a much stronger potential drug target than other chemokines. Fractalkine is also of significant interest as it is expressed within the CNS where it is released from neurons and the receptor is expressed on glial cells. These features have prompted research into the potential role of fractalkine in pain; for a review see Abbadie *et al* (2003).

Intrathecal injection of fractalkine results in allodynia and hyperalgesia in rats and this is inhibited by administration of either the glial antagonist, minocycline, or anti-IL1 $\beta$  demonstrating that the effects of fractalkine are, in part, regulated by other inflammatory mediators (Milligan *et al.*, 2005, Han and Neugebauer, 2005). Furthermore, fractalkine over-expression results in the development of hyperalgesia and allodynia in rats with a concomitant increase in IL-1 $\beta$  in the CSF indicating that there may be a positive feedback loop of glial activation and neuronal release of fractalkine contributing to the increased pain response (Langer *et al.*, 2006).

In animal pain models fractalkine has been shown to increase in the spinal cord and DRG in rats in the neuropathic CCI and SNL models but not the inflammatory CFA model (Han and Neugebauer, 2005, Milligan *et al.*, 2005, Axen and Porath, 1966). Interestingly the fractalkine receptor has been

shown to increase in the ipsilateral spinal cord in an inflammatory model of CFA induced mono-arthritis and this increase was associated with glial activation. The activation of glial cells was attenuated by administration of a CX3CR1 neutralising antibody as was the increased hyperalgesia and allodynia, however, the behavioural changes were only reversed for three days while the glia remained inactivated.

The regulation of fractalkine release is controlled by microglial release of the protease, cathepsin-s, which cleaves the mucin stalk holding the chemokine to the cell membrane. Inhibition of cathepsin-s reverses symptoms of neuropathic pain (Wu *et al.*, 2005, Clark *et al.*, 2009, Clark *et al.*, 2007).

Another chemokine that has sparked interest in the field of chronic pain is CXCL1, also known as growth-related oncogene or GRO. CXCL1, best known for its role in cancer, is up regulated in paw tissue in a model of the adaptive immune response where it was shown to be upregulated in response to increased TNF $\alpha$  expression. CXCL1 release also regulated hypernociception in immunised animals in a prostanoid and amide dependant manner (Cunha *et al.*, 2008). CXCL1 is also upregulated in the DRG following in both neuropathic and inflammatory pain models and overnight incubation of small diameter sensory neurons with this chemokine resulted in protein dependant increase Na currents and increased expression of Na1.1, 1.7 and 1.8 subunits. Taken together, these results point to a key regulatory role for CXCL1 in nociception.

There is a known link between inflammation and hypoxia, for example in stroke many inflammatory mediators are released following ischaemic attack. Both the cytokines (IL-1 $\beta$  and TNF $\alpha$ ) and chemokines (CXCL1 and CX3CL1) have been implicated in stroke (Wang *et al.*, 2007) and hypoxia in the periphery has been associated with inflammatory pain conditions such as rheumatoid arthritis, CRPS and FM (Peters *et al.*, 2004, Koban *et al.*, 2003, Jeschonneck *et al.*, 2000).



### **6.1.2 Aims**

As discussed above there is a significant role for inflammation in the development and maintenance of pain states which has been demonstrated in the periphery and the spinal cord of the CNS. There is limited information about the role of inflammation in the brain although it is evident that changes do occur in higher brain centres in chronic pain conditions (see Chapters 2 and 4).

The aims of this chapter were to measure the gene expression of various inflammatory mediators throughout the rat brain in response to the persistent inflammatory pain model used in Chapter 5 and to see if there is any evidence of hypoxia in response to inflammation by measuring hypoxia inducible factor 1 $\alpha$  (HIF1 $\alpha$ ). The brain was divided into 6 sections (see figure 25) to allow comparisons of regions on both the left (contralateral to inflamed paw) and right (ipsilateral to inflamed paw) sides of the brain. mRNA expression of cytokines (IL-1 $\beta$ , TNF $\alpha$ ) and chemokines (CX3CL1, CXCL1) along with the chemokine receptor CX3CR1 and HIF1 $\alpha$  were measured at acute (6 hours) subacute (24 and 72 hours) and persistent (7 and 14 days) timepoints as well as in non-injected control animals.

## **6.2 Materials and Methods**

### **6.2.1 RNA extraction**

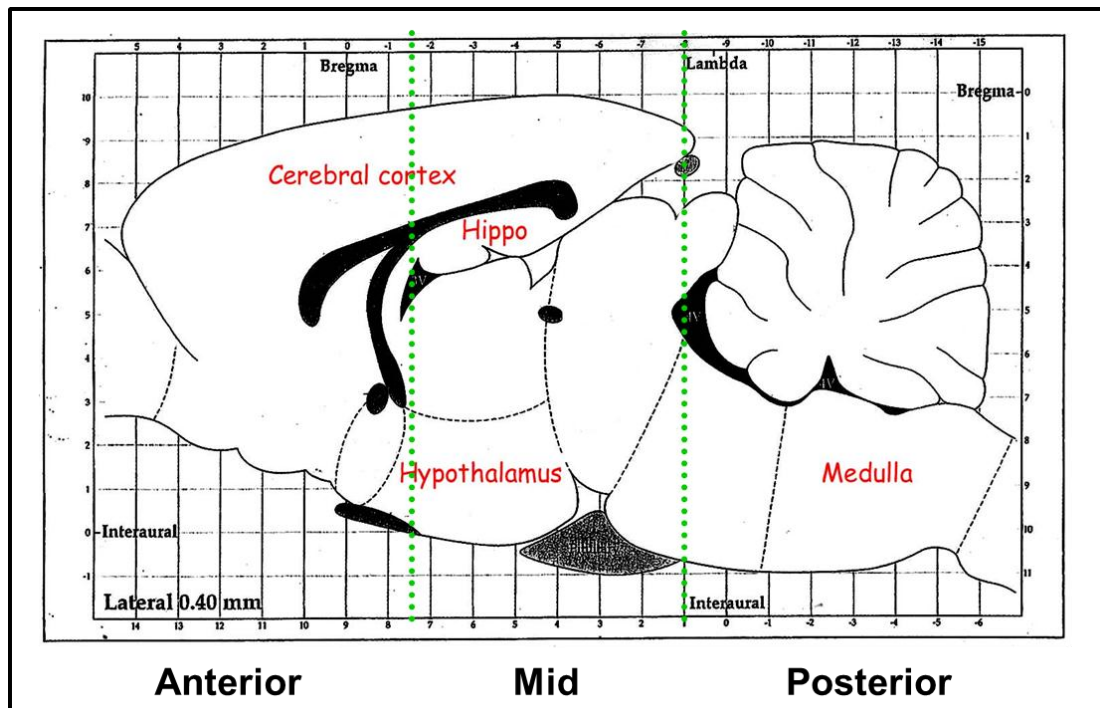
#### **6.2.1.1 Materials**

DEPC, chloroform, ethanol and isopropanol (Fisher Scientific, Leics, UK). TRIzol reagent and DEPC treated water (Invitrogen Ltd, Paisley, UK).

#### **6.2.1.2 Methods**

All procedures were carried out using DEPC treated apparatus and containers in order to prevent contamination by RNases and DNA. The

protocol used is adapted from the TRIzol manufacturer's instructions and based on that of Chomczynski and Sacchi (1987).



**Figure 25 Rat Brain Sections for PCR analysis.** Brains were separated into left and right sections and each section divided into anterior, mid and posterior using a brain atlas and matrix as a guide.

Prior to RNA extraction brains were cut into six blocks, firstly they were separated in to left and right hemisphere and then each hemisphere divided into three blocks, (see figure 25). Sections were stored in individual eppendorf tubes at -70°C until further analysis.

Brain blocks were allowed to fully defrost on wet ice and then homogenised with 1ml TRIzol per 100mg tissue using a hand held glass homogeniser and the resultant solution transferred to 1ml eppendorf tubes. 200µl chloroform per 1ml TRIzol used was added to each tube and the solution shaken hard for 15 seconds then incubated at room temperature for three minutes. The solution was then centrifuged for 15 minutes at 12,000g and 4°C separating the homogenate into three layers, the aqueous phase from each tube was transferred into individual fresh tubes taking great care not to disturb the layers underneath. The remaining fractions, which contain DNA and protein,

were stored at -70°C for potential future use. 0.6ml of isopropanol per 1ml of TRIzol used was added to the aqueous fractions which were mixed and then incubated at room temperature for ten minutes to allow precipitation of RNA which was then separated by centrifugation at 12,000g and 4°C for ten minutes. After centrifugation a pellet was usually visible at the bottom of the tube, the supernatant was decanted and the pellet washed twice with 1ml of cold 75% ethanol by vortexing and then centrifuging for five minutes at 7,500g and 4°C. The ethanol was decanted and the pellet air dried at room temperature for ten minutes and then resuspended in 20µl DEPC-treated water per 1ml trizol used. To ensure the RNA was fully dissolved the solution was vortexed and then heated at 60°C for ten minutes before being immediately cooled on wet ice. RNA was quantified by measuring adsorption at A260 and purity measured by the A260/A280 ratio prior to being stored at -70°C for future use.

## **6.2.2 Reverse Transcription**

### **6.2.2.1 Materials**

In-Prom II RT kit and DEPC treated water (Promega UK, Southampton, UK)  
DNase I amplification grade kit (Invitrogen Ltd, Paisley, UK).

### **6.2.2.2 Methods**

This protocol is based on the manufacturer's suggested method for using the In Prom II RT kit (Promega) and modified to enable addition of Invitrogen DNase I (Invitrogen). Briefly, for one reaction 1.6µg RNA (isolated as in 6.2.1 RNA extraction), 1.6µl 10X DNase buffer solution, 1.6µl DNase and DEPC-water to make a total volume of 16µl were added to a 200µl PCR tube. A water control was also set up containing water instead of RNA. The tubes were incubated at room temperature for fifteen minutes and then 1.6µl 25mM EDTA added and the tubes heated at 65°C to stop the DNase reaction, the tubes were then placed on ice prior to use in the RT reaction.

For the RT reaction the relevant volume of mastermixes were made up as per the amounts in table 4 and divided between reaction tubes (10.2µl per tube) to which 9.8µl of DNase treated RNA had been added.

	Experimental	RT negative
H <sub>2</sub> O	1.3µl	2.3µl
Buffer	4.0µl	4.0µl
MgCl <sub>2</sub>	2.4µl	2.4µl
dNTPs	1.0µl	1.0µl
RNasin	0.5µl	0.5µl
Reverse Transcriptase	1µl	X

**Table 4 Volumes of reagents required for each RT reaction.** The “RT negative” column is for the negative controls which contain no enzyme and the “Experimental” column for the regular samples and the water control.

Samples were then placed in a thermocycler and the following cycle run:

**Anneal:** 25°C 5min  
**Extend:** 42°C 1 hour  
**Inactivate:** 70°C 15min  
**Hold:** 4°C

The resultant product was then placed on ice prior to use in a PCR reaction or stored at -70°C until future use.

## 6.2.3 Primer Design and Optimisation

### 6.2.3.1 Primer Design

The mRNA FASTA sequence of interest was identified by searching Entrez Gene, the Primer 3 program was then used to generate primer sequences using the following constraints:

Product size ranges: 301-400bp, 401-500bp  
 Primer T<sub>M</sub>: opt 60°C  
 Primer length: opt 20

A BLAT search of the mRNA sequence was used to ensure specificity and identify exon/intron boundaries. This information was cross-referenced with the primer 3 output and primers were chosen that spanned to different introns to ensure that any contamination from genomic DNA would be apparent in the PCR product. A final check on specificity was made using the UCSC in-silico PCR facility.

Name	Genebank #	Sequence	TM	TA	PCR Cycles	Product Size (KDa)
$\beta$ actin F	NM_031144	TGTCACCAACTGGGACGATA	60	61	30	392
$\beta$ actin R		TCTCAGCTGTGGTGGTGAAG	60	61	30	
Hif 1 $\alpha$ F	NM_024359	TGCTCATCAGTTGCCACTTC	60	61	30	305
Hif 1 $\alpha$ R		CATGGTCACATGGATGGGTA	60	61	30	
CXCL1F	NM_030845	AGCTCCAGCACTCCAGACTC	60	55	30	364
CXCL1R		GCCATCGGTGCAATCTATCT	60	55	30	
IL1 $\beta$ F	NM_031512	CAGCAGCATCTCGACAAGAG	60	55	30	362
IL1 $\beta$ R		GGGATTTTGTCGTTGCTTGT	60	55	30	
TNF $\alpha$ F	NM_012675	GAGGTCAACCTGCCCAAGTA	60	55	30	263
TNF $\alpha$ R		CGTGTGTTTCTGAGCATCGT	60	55	30	
CX3CR1F	NM_133534	CAGGACCTCACCATGCCTAC	60	55	30	657
CX3CR1R		GATGCGGAAGTAGCAAAAGC	60	55	30	
CX3CL1F	NM_134455.1	CTCTGCTGGCGGGTCAGCAC	60	55	35	491
CX3CL1R		GCTCTGAGGCTTGGCGGCAA	60	55	35	

**Table 5 Primers designed and used in this work.** F forward primer, R reverse primer TM/TA primer melting/annealing temperature °C.

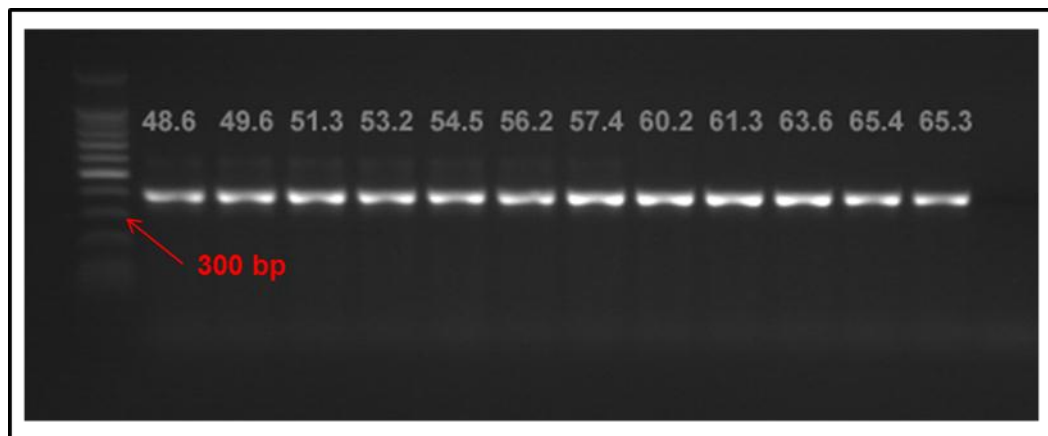
### 6.2.3.2 Primer Optimisation

Although the primer design process includes specific parameters for the PCR protocol used it is sometimes necessary to further optimise the protocol for individual primer pairs. The two parameters optimised here were number of cycles and annealing temperature.

If very little product is visible it is possible to increase the production by increasing the number of PCR cycles. Care must be taken as too many cycles can result in amplification of non-specific products and over exposure

of the gel. Here 30 cycles were used for all primer pairs other than CXCL1 where this was not sufficient. Initially 40 cycles was tried, however, this resulted in amplification of non-specific products. 35 cycles produced a much cleaner gel where it was still possible to measure differences between bands.

Gradient PCR was used to optimise the annealing temperature for primer pairs where non-specific products or inconsistent results were an issue. For most primer pairs an annealing temperature of 5°C less than the melting temperature is used, which in the case of the primers used here is 55°C. Hif-1 $\alpha$  and  $\beta$  actin primer pairs generated non-specific products at this temperature and gradient PCRs were used to optimise an annealing temperature from ~50°C-65°C, (see figure 26).



**Figure 26  $\beta$  actin temperature gradient PCR.** The same sample was subjected to PCR as described below with a variety of annealing temperatures, as indicated above each lane (°C). Non-specific product is apparent in the first 8 lanes but disappears at higher temperatures, an annealing temperature of 61°C was chosen for this primer pair.

## 6.2.4 PCR Amplification

### 6.2.4.1 Materials

GoTaq green mastermix and DEPC treated water (Promega UK, Southampton, UK). Custom primers (Sigma-Aldrich co Ltd, Poole, UK).

### 6.2.4.2 Methods

The Promega GoTaq mastermix was used for all PCR reactions and the method was carried out according to the manufacturer's protocol. Briefly, cDNA template was allowed to defrost on ice and then 1µl of each sample added to a PCR tube, 1µl of water was added to a final tube to act as a control. A mastermix was made containing the following for each sample:

12.5µl GoTaq green  
0.25µl forward primer  
0.25µl reverse primer  
11µl DEPC water

24µl of mastermix was added to each sample tube and mixed resulting in a total reaction volume of 25µl. Tubes were then placed in a thermocycler and the following cycle run:

Initial Denaturation: 94°C 2 min

Denaturation: 95°C 45 sec	}	30 cycles*
Annealing*: 55°C 45 sec		
Extension: 72°C 45 sec		

Final extension: 72°C 5 min

Hold: 4°C

Parameters marked with an asterisk were optimised for individual primer pairs, see primer section for details.

Following PCR amplification the samples were immediately placed on ice, centrifuged briefly to collect any condensate that had formed, and then stored at -20°C until require for further analysis.

## **6.2.5 Gel Electrophoresis and Semi-Quantitative Analysis**

### **6.2.5.1 Materials**

Agarose, tris base, boric acid, 0.5M EDTA (pH 8.0), ethidium bromide solution (10mg/ml) and 100 base pair DNA ladder (Fisher Scientific, Leics, UK).

### **6.2.5.2 Methods**

A 1.2% agarose gel was made with 0.5X TBE and 8µl ethidium bromide per 100ml gel. The samples were defrosted on ice, centrifuged briefly to collect any condensate, and 10µl loaded per well. A DNA ladder was loaded into the first lane of every row for size comparison and βactin (5µl per well) was used as a control. The gel was run at 100mV in 0.5X TBE buffer until the dye front was level with the base of the gel. Bands were visualised using UV and quantified by densitometric analysis using the Scion Image program.

## **6.2.6 Statistical Analysis**

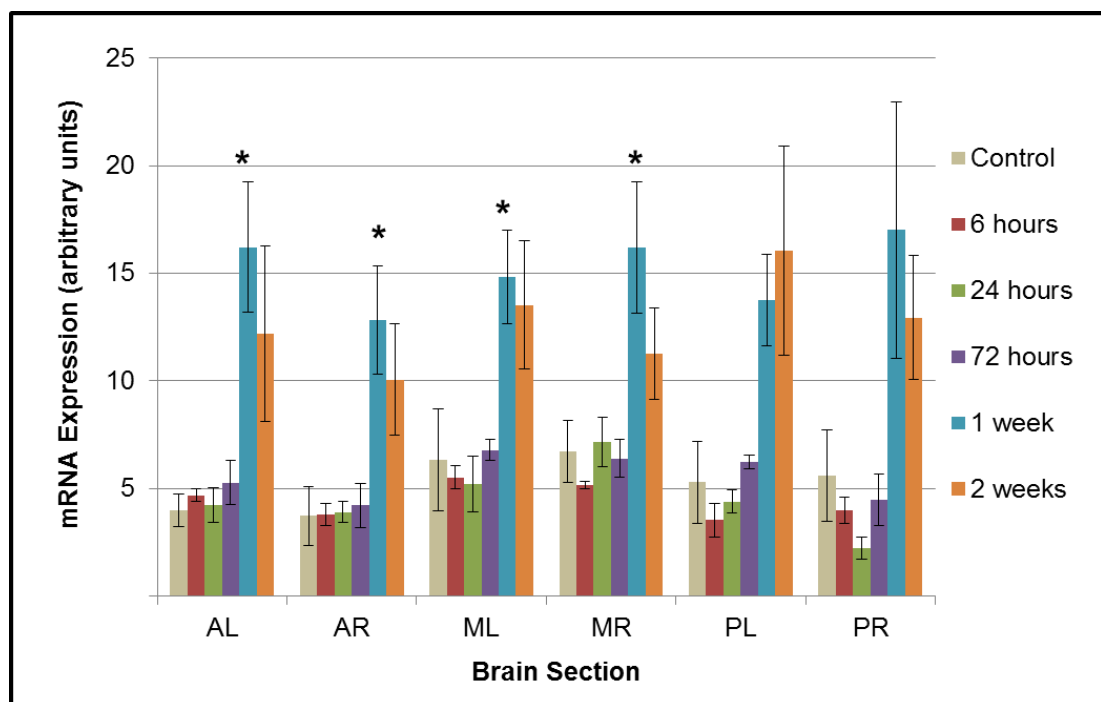
All results were normalised to βactin control measurements and compared using analysis of variance (ANOVA) followed by Tukey's honestly significant difference test (Tukey's HSD) for post-hoc analysis. All analysis was performed using SPSS and  $p < 0.05$  was considered significant. Graphs display mean values and error bars represent +/- the standard error of the mean (SE).



## 6.3 Results

### 6.3.1 IL1 $\beta$ Expression

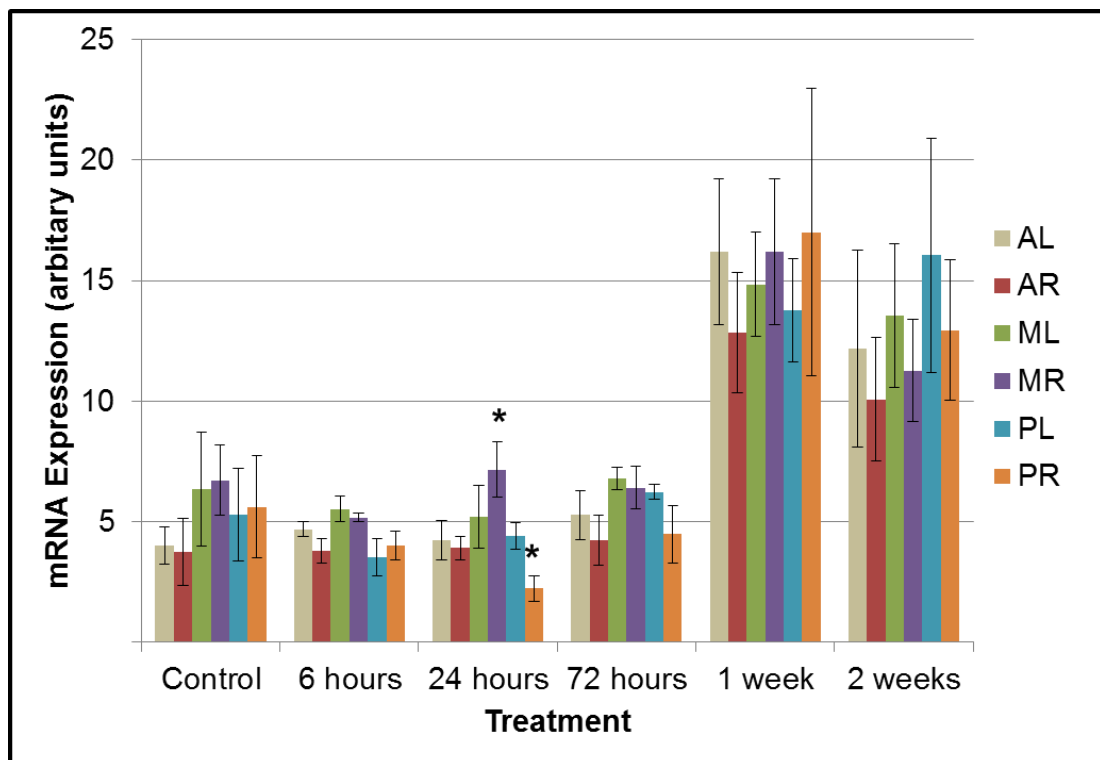
IL1 $\beta$  mRNA expression was greatly increased in all sections of the brain at one week post-injection compared with non-injected controls and 6, 24, and 72 hour CFA treated animals. This difference was significant in all anterior and mid regions of the brain but not in the posterior regions which just failed to reach significance. At 14 days post-injection there is an overall drop in expression from 7 days although IL1 $\beta$  expression in all sections remained higher than normal and 6, 24 and 72 hour time-points. A decrease in expression is also seen in the back of the brain at 6 and 24 hours post-injection (see figure 27).



**Figure 27 IL1 $\beta$  mRNA expression in CFA treated animals and non-injected controls by time point.** There is a large increase in IL1  $\beta$ mRNA expression at 7 and 14 days post injection. In the anterior and middle of the brain expression dropped at 6 hours before increasing at later time points.  $n=4$  \*  $p<0.05$  compared with all other groups in that section A anterior, M mid, P posterior, L left, R right.

When looking at the pattern of expression throughout the brain by time point the IL1 $\beta$  mRNA expression profile remains fairly consistent at each time point. There were no significant differences between sections at any time

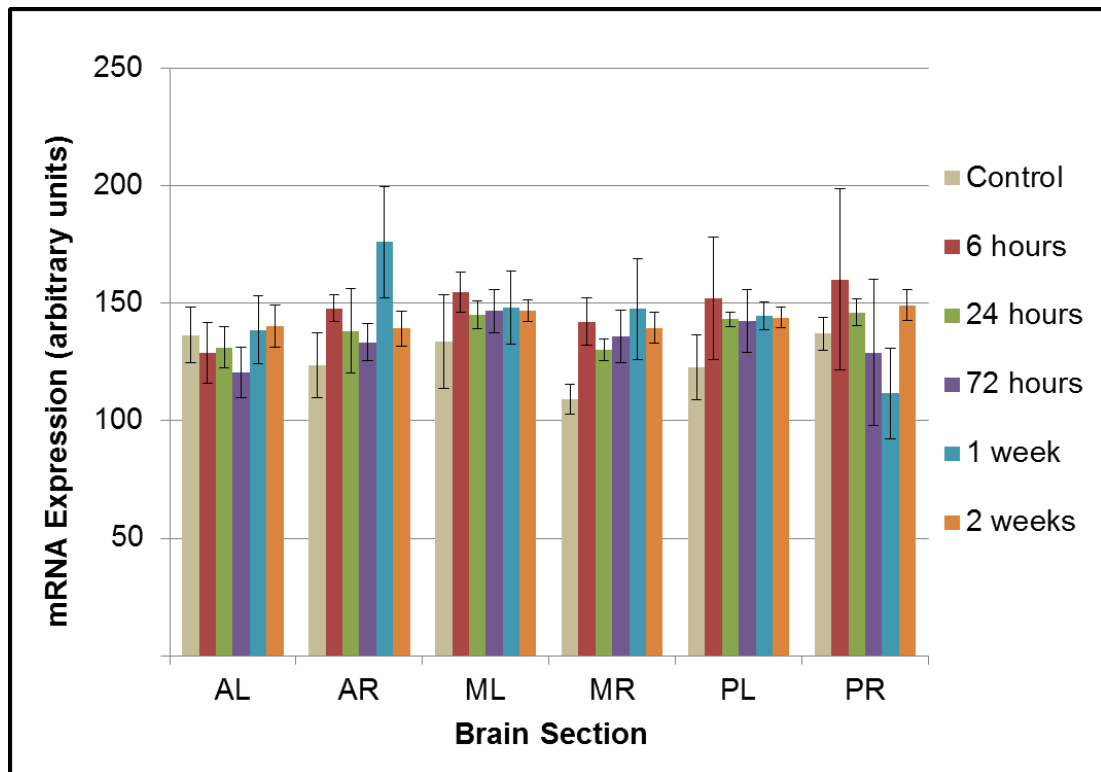
point other than 24 hours where the mid-right section expressed significantly higher levels than those in the right posterior. Interestingly at 14 days post-injection, although the relative expression from posterior to anterior regions of the brain is similar to previous time points, the left side of the brain expresses more IL1 $\beta$  mRNA than the right in all three regions (see figure 28).



**Figure 28 IL1 $\beta$  mRNA expression in CFA treated animals and non-injected controls by brain region.** There are no significant differences according to brain region other than at 24 hours where the mid-right section of the brain expressed significantly more IL1 $\beta$ mRNA the back right. Expression was higher at 1 and 2 weeks than non-injected controls and the three earlier injected time points. n=4 \* p<0.05 for difference between MR and BRF front, A anterior, M mid, P posterior, L left, R right

### 6.3.2 Hif1 $\alpha$ Expression

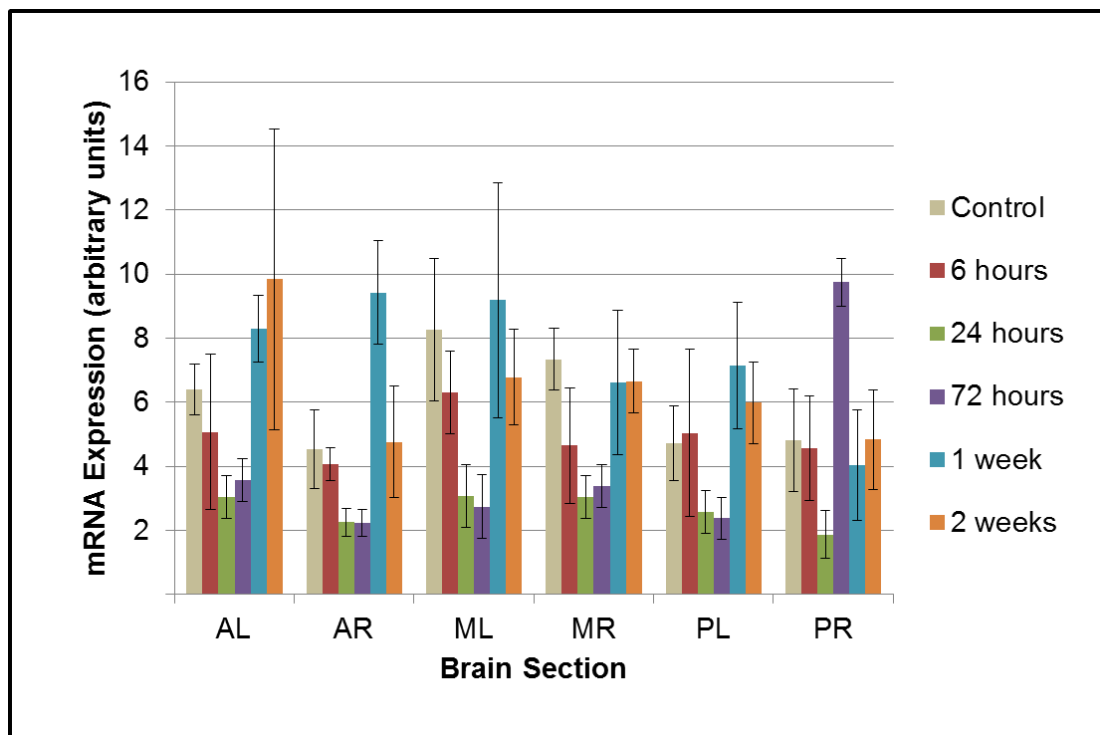
There were no significant differences in Hif1 $\alpha$  mRNA expression when assessed relative to time point or brain region (see figure 29).



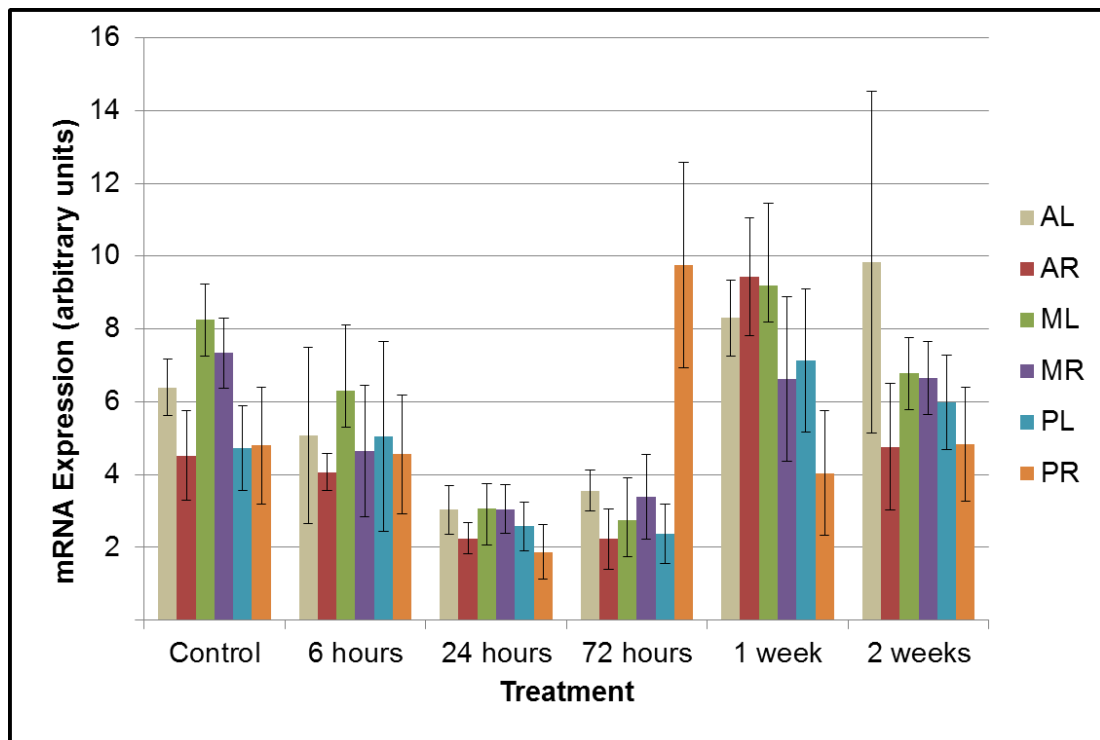
**Figure 29 Hif1 $\alpha$  mRNA expression in CFA treated animals and non-injected controls by time point.** There was no significant difference in Hif1 $\alpha$  mRNA expression at any time point although there is a trend towards an increase in CFA treated animals. n=4 A anterior, M mid, P posterior, L left, R right

### 6.3.3 TNF $\alpha$ Expression

There was a slight decrease in TNF $\alpha$  mRNA expression from control to six hours post-injection which became more marked at 24 hours and remained low at 72 hours other than in the back right of the brain where there was a large increase in expression at 72-hours post-injection. At 7 days expression increased to just above baseline in all sections other than the right posterior where there was a drop to just below baseline (see figure 30).



**Figure 30 TNF $\alpha$  mRNA expression in CFA treated animals and non-injected controls by time point.** In all areas of the brain there was a drop in TNF $\alpha$  mRNA expression at 24 hours post-injection, levels remained low at 72 hours in all areas except the right posterior region where there was a large increase. At one week there was a trend to an increase in the anterior and mid sections of the brain. n=4 A anterior, M mid, P posterior, L left, R right

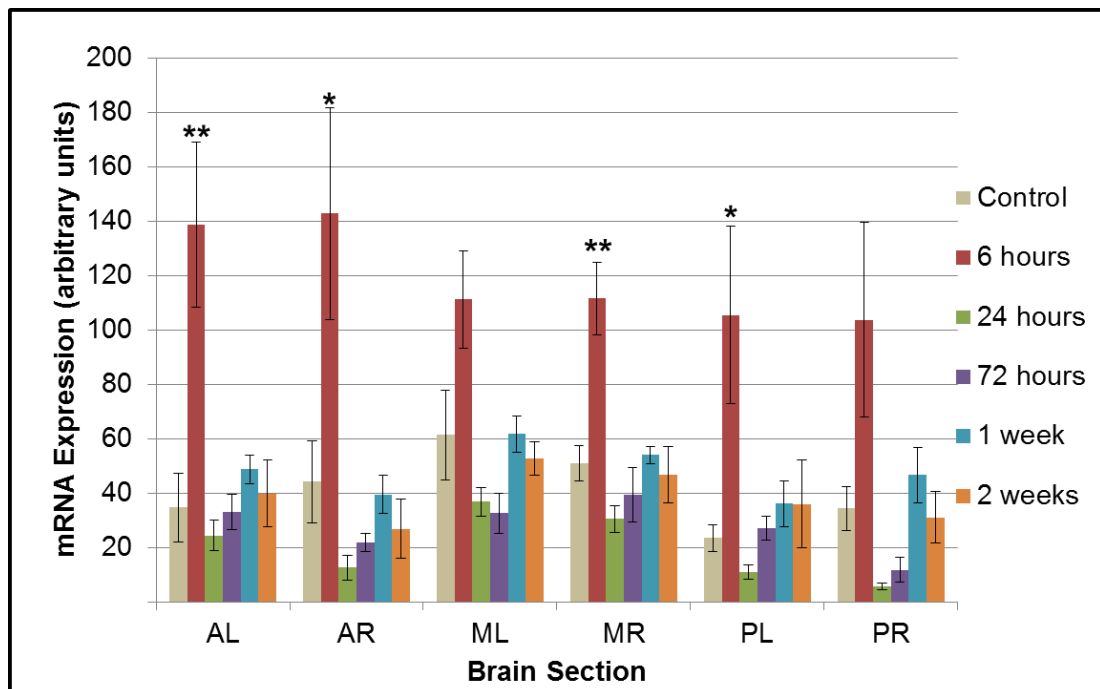


**Figure 31 TNF $\alpha$  mRNA expression in CFA treated animals and non-injected controls by brain region.** There is a drop in expression of TNF $\alpha$  mRNA in all areas of the brain at 24 and 72 hours other than the posterior right at 72 hours. At 1 and 2 weeks expression increases to at least baseline. n=4 + A anterior, M mid, P posterior, L left, R right.

When TNF $\alpha$  mRNA data are expressed by brain section it can be seen that levels vary slightly throughout the control brain, with greater expression in the middle of the brain. At six hours there is a slight decrease in expression which becomes much more evident by the 24 hour time point; levels remain low in all brain areas at 72 hour post injection other than in the posterior right region which has much higher expression than all other sections at this time point. At one and two weeks post-injection expression returns to roughly the levels seen in the control groups other, the front regions showing slightly higher levels at 7 days. There was no significant difference between expression in the left and right sides of the brain (see figure 31).

### 6.3.4 CXCL1 Expression

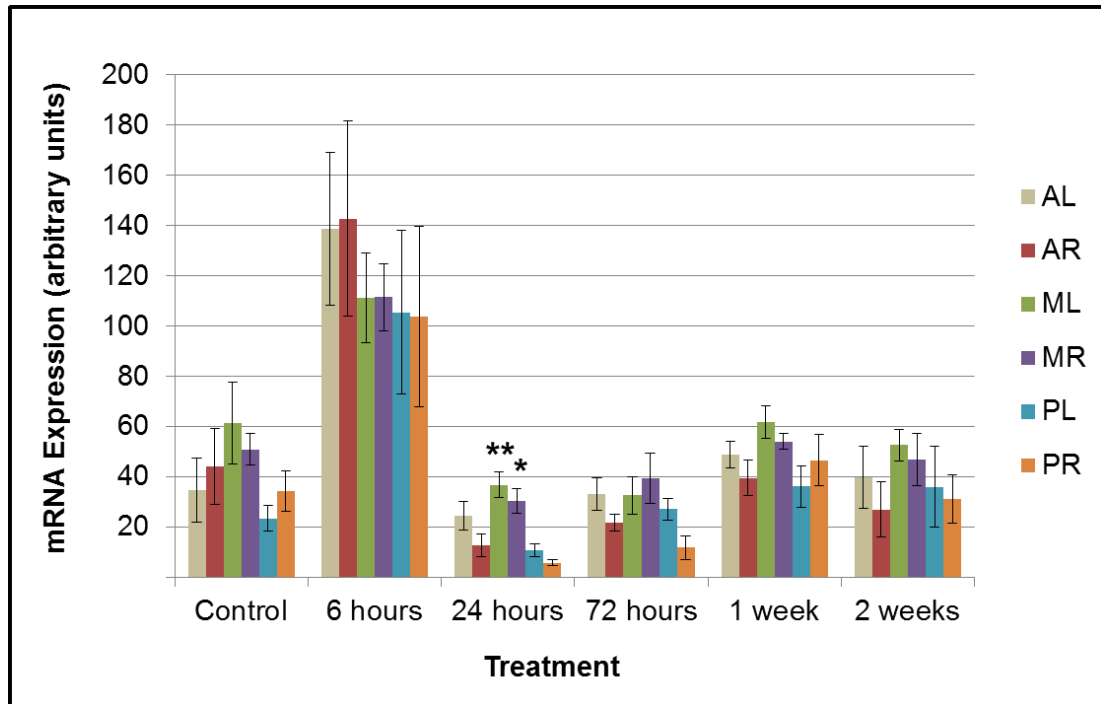
CXCL1 expression was greatly increased at 6 hours post-injection in all brain regions, the largest increase being in the anterior brain regions and the lowest in the middle. At 24 hours post-injection expression in all brain regions dropped to below baseline increasing slightly at 72 hours but remaining significantly lower than the expression at 6 hours at both time points. At the later time points of 1 and 2 weeks CXCL1 expression returned to baseline in all brain regions but remained significantly lower than expression at 6 hours in the anterior and mid brain regions (see figure 32).



**Figure 32 CXCL1 mRNA expression in CFA treated animals and non-injected controls by time point.** There is a large increase in CXCL1 mRNA expression in all brain regions at 6 hours post injection this was significantly greater than control in anterior regions and in mid-right and posterior-left sections.  $n=4$  \*  $p<0.05$  \*\*  $p<0.01$  A anterior, M mid, P posterior, L left, R right.

There is a consistent profile of expression throughout the brain sections at all time points, other than six hours post-injection, with mid brain regions expressing more CXCL1 mRNA than the anterior and posterior regions. This difference was significant at 24 hours with the left middle section expressing more than the front right and both back sections ( $p<0.01$ ) and the right middle section expressing more than the back right ( $p<0.01$ ) and left

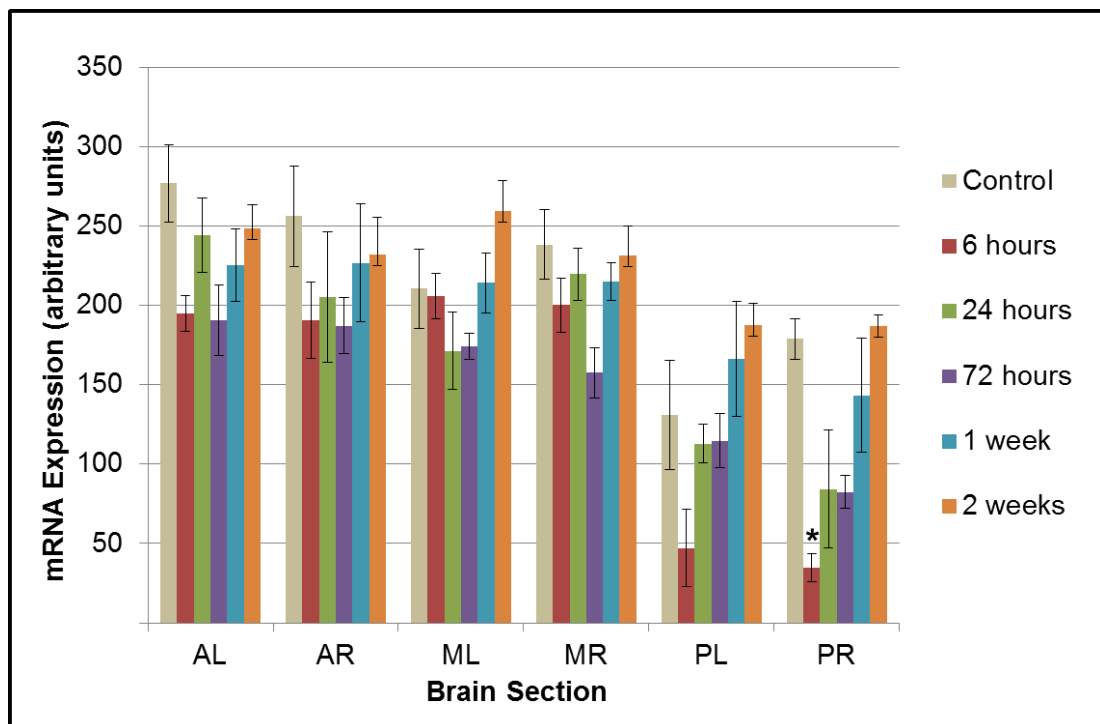
( $p < 0.05$ ). In CFA treated rats at the 6 hour time point, where CXCL1 expression is significantly increased (see figure 32), the profile of expression changes with higher levels in the anterior regions of the brain, decreasing in the middle and lowest in the posterior regions (see figure 33).



**Figure 33 CXCL1 mRNA expression in CFA treated animals and non-injected controls by brain region.** Expression peaked at 6 hours post-injection decreasing to below baseline at 24 and 72 hours before returning to baseline at 1 and 2 weeks. At 2 hours post-injection expression in the middle two sections was significantly higher than in anterior and posterior regions, see text for further explanation.  $n=4$  \*  $p < 0.05$  \*\*  $p < 0.01$  A anterior, M mid, P posterior, L left, R right.

### 6.3.5 CX3CL1 Expression

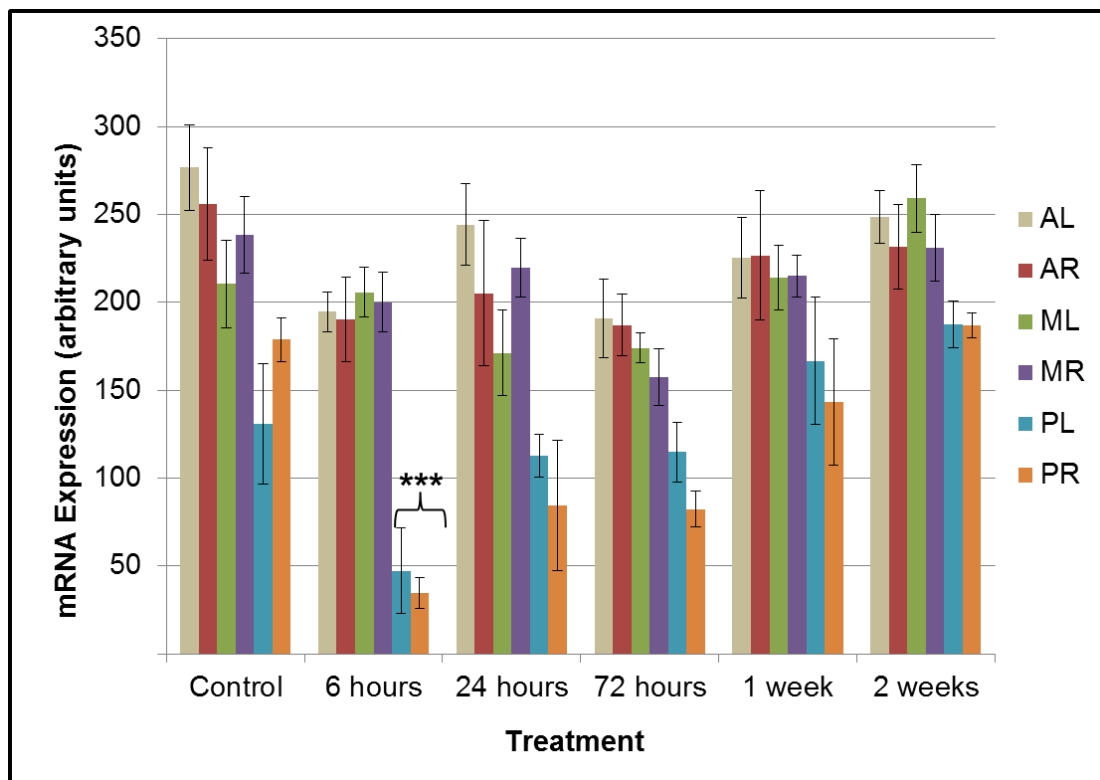
CX3CL1 mRNA expression was decreased in all areas of the brain post-injection but most significantly in the posterior regions where there was a very large drop in expression at 6 hours post-injection which gradually increased at each time point returning baseline in the right posterior region and exceeding baseline in the left (see figure 34).



**Figure 34 CX3CL1 mRNA expression in CFA treated animals and non-injected controls by time point.** In all brain regions there was a drop in CX3CL1 mRNA expression post-injection at 6 hours which then increased to baseline or above. This was most evident in the posterior regions of the brain the 6 hour time point in the back left was significantly lower than control.  $n=4$  \*  $p<0.05$  A anterior, M mid, P posterior, L left, R right.



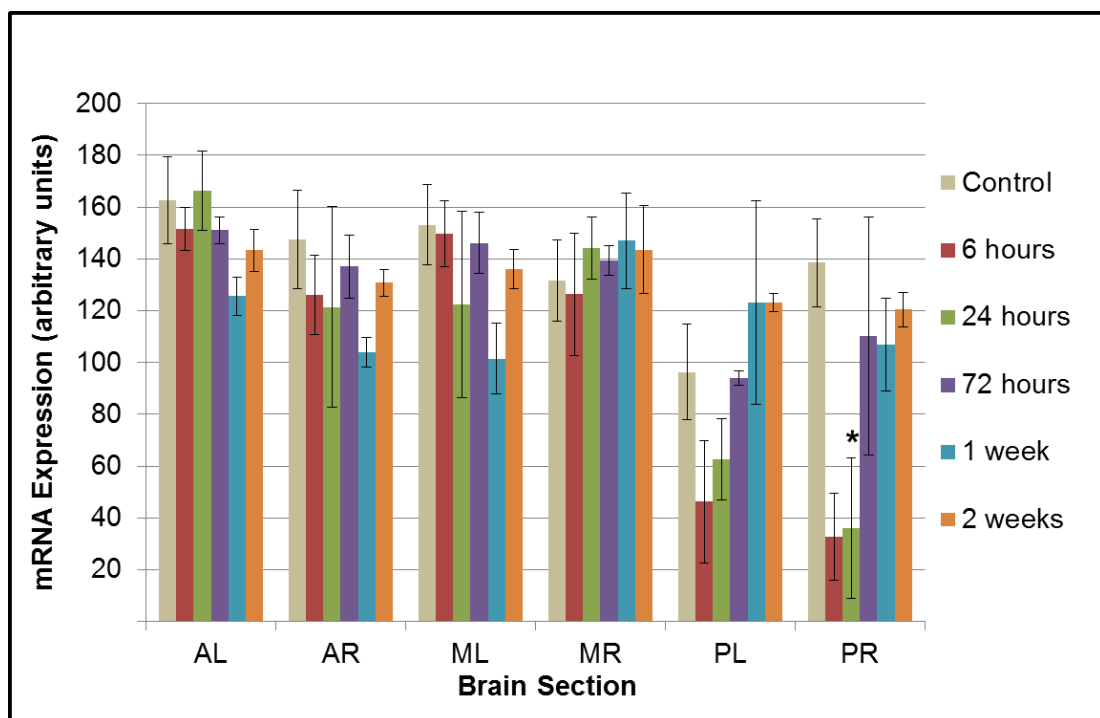
Across the brain, CX3CL1 mRNA expression decreased from posterior to anterior regions in both control and all CFA treated animals. At 6 hours this difference is most significant ( $p < 0.001$ ) where both left and right back regions of the brain express significantly lower levels than all other areas. No significant difference was seen between left and right regions, however at 72 hours, 1 week and 2 weeks post-injection left brain regions expressed higher levels of CX3CL1 mRNA than the corresponding right regions (see figure 35).



**Figure 35 CX3CL1 mRNA expression in CFA treated animals and non-injected controls by brain section.** At all time points CX3CL1 expression is lower in the posterior regions of the brain compared with the middle and anterior. This difference was most evident at six hours where the difference in expression was significantly lower in both back regions compared with all other sections. N=4 \*\*\*  $P < 0.001$  A anterior, M mid, P posterior, L left, R right.

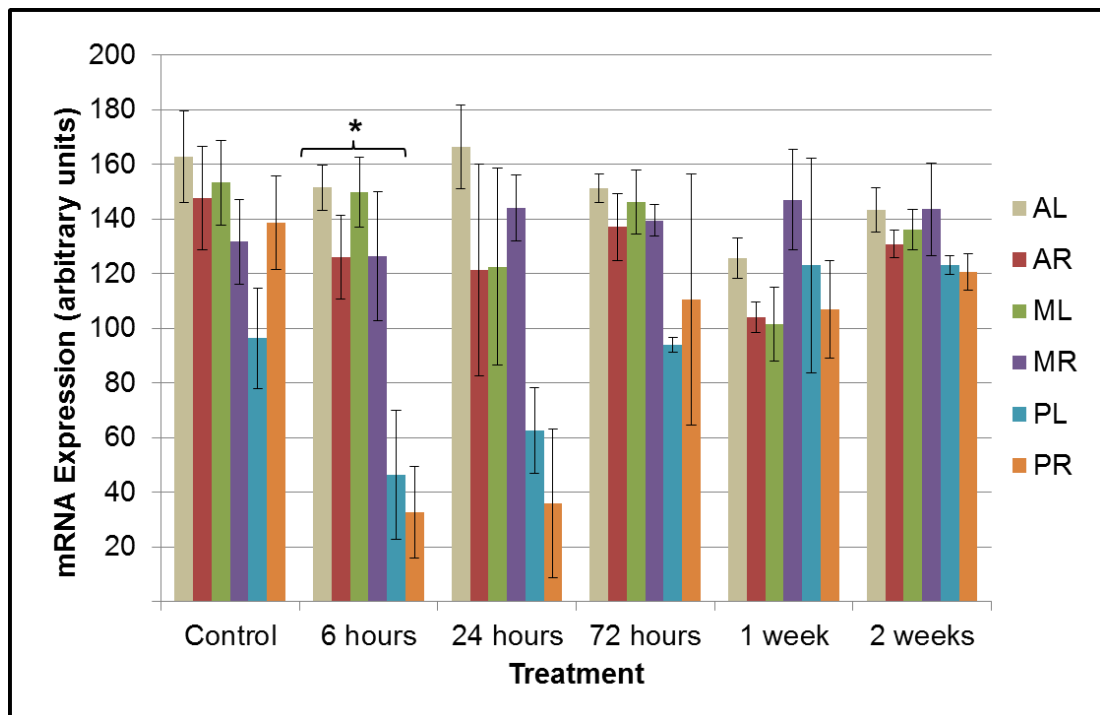
### 6.3.6 CX3CR1 Expression

In both anterior sections of the brain and in the middle left region the expression of CX3CR1 mRNA tended to decrease over time post-injection with the minimum expression seen at 1 week. Conversely in the middle right brain section expression increased post injection with the highest expression seen at 7 days. In both anterior regions of the brain there was a large decrease in expression of CX3CR1 at 6 hours post-injection which remained relatively low at 24 hours before returning to (right section) or slightly exceeding baseline (left section) this decrease was significant only at the 24 hour time point in the right anterior region of the brain when compared with control ( $p < 0.05$ ) (see figure 36).



**Figure 36 CX3CR1 mRNA expression in CFA treated animals and non-injected controls by time point.** Expression was decreased at 6 and 24 hours post-injection in the anterior of the brain increasing back to baseline (right section) or above (left section). In the right anterior section expression at 24 hours was significantly lower than in control ( $p < 0.05$ ).  $n=4$  \*  $p < 0.05$  A anterior, M mid, P posterior, L left, R right.

In non-injected control animals expression does not differ greatly between brain regions, however at 6, 24 and 72 hours post-injection there is a larger drop in expression, most evident at the earlier time points. At 6 hours this difference is significant in both left and right back brain sections when compared with left front and middle regions ( $p<0.01$ ) and right front and middle sections ( $p<0.05$ ). At 24 hours the difference only reached significance between the front left and back right sections ( $p<0.01$ ). The profile of receptor gene expression at one and two weeks did not differ greatly from control and no significant differences were found between left and right regions (see figure 37).



**Figure 37 CX3CR1 mRNA expression in CFA treated animals and non-injected controls by brain section.** At 6, 24 and 72 hours post-injection expression is lower in the back of the brain when compared with the middle and front regions. At six hours this difference is significant in both back regions when compared with all other brain regions. N=4 \*  $p<0.05$  A anterior, M mid, P posterior, L left, R right.

## 6.4 Discussion

This chapter set out to investigate the expression of a variety of inflammatory mediators in a model of persistent inflammatory pain, the expression profile identified is discussed below.

### 6.4.1 IL1 $\beta$ Expression

IL1 $\beta$  expression was increased at 7 and 14 days post-injection in all brain regions, although this failed to meet significance in the anterior sections of the brain and at 14 days. At 6 and 24 hours post-injection there is a decrease in IL1 $\beta$  expression in the anterior of the brain which was significant in several areas at 24 hours. These results are not totally in accordance with previously published data where an increase in IL1 $\beta$  mRNA was seen at earlier time points in both neuropathic and inflammatory models (Raghavendra *et al.*, 2004, Uceyler *et al.*, 2008). This variation may be due to several factors, slightly different time points were used in this study compared with previous studies and it is possible that increases in expression at earlier time points would have been seen in the current model.

The increase in IL1 $\beta$  expression corresponds with the increased paw swelling seen here at 7 and 14 days (see figures 23 and 24) and the increased allodynia described in previous work (Stein *et al.*, 1988, Raghavendra *et al.*, 2004), however there was no increase in IL1 $\beta$  expression at the 6 hour time point where swelling and nociceptive responses show an initial peak. This suggests that IL1 $\beta$  may be involved in the regulation of brain processes in longer term pain but not at the acute stage.

One suggestion might be that the drop in expression in the back of the brain at 6 and 24 hours is a form of endogenous analgesia in response to the rapid increase in pain and swelling in the acute stages. The increase in expression at the other time points may be the result of continued nociceptive input stimulation and increase in IL1 $\beta$  expression leading to changes in structure

and function within these brain areas. IL1 $\beta$  has been demonstrated to play a role in LTP and it, as such, may be establishing a pain “memory” in the brain which may increase the potential for chronic pain to develop (Wang *et al.*, 2000, Zeise *et al.*, 1992).

#### **6.4.2 Hif1 $\alpha$ Expression**

No significant changes were seen in Hif1 $\alpha$  mRNA expression although there were some minor increases in CFA treated animals compared with controls with 22 out of 30 time points having higher expression than their respective non-injected controls. This increase could be due to the increased inflammation seen in the brain as Hif1 $\alpha$  had been shown to be upregulated by inflammation in the absence of hypoxia (Olson and van der Vliet, 2011). It is also possible that hypoxia is present, but in discrete areas of the brain which were not identified by the methods used here.

#### **6.4.3 TNF $\alpha$ Expression**

TNF $\alpha$  mRNA expression decreased from 6-72 hours post-injection in all brain areas other than the anterior right section where there was a large peak in expression at 72 hours (see figure 31) It is difficult to explain this result since any increase that wasn't present on both sides of the brain would be predicted to occur on the contralateral side to the injected paw. One suggestion might be that this increase in TNF $\alpha$  is the result of top-down regulation, i.e. an increase in activity in a descending modulatory pathway which would occur on the ipsilateral side having potential nociceptive or indeed analgesic effects (see figure 3).

Further support for a role for TNF $\alpha$  in descending regulatory pathways comes from the increase in expression in the front brain regions at 7 and 14 days, these regions contain many of the nuclei responsible for top down regulation.

#### 6.4.4 CXCL1

This is the first evidence for an increase in CXCL1 expression in the brain in chronic pain. The highly significant increase at 6 hours suggests a role for this chemokine in the acute response. CXCL1 may play a more significant role in the anterior of the brain since the increase in expression is greatest here, differing from the pattern of expression seen at other time points where the middle brain areas express the highest levels (see Figure 33).

In the periphery, TNF $\alpha$  has been demonstrated to induce CXCL1 expression (Cunha *et al.*, 2008) however, this is not the case here where, in agreement with results seen in neuropathic pain models, there is a decrease in TNF $\alpha$  expression at early time points.

The peak in CXCL1 expression at 6 hours corresponds with the first peak seen in paw swelling (see figure 24) and hyperalgesia and allodynia reported elsewhere (Stein *et al.*, 1988). However, CXCL1 levels drop massively at the 24 hour time point, particularly in the most anterior and posterior regions of the brain, whereas oedema and nociceptive measurements remain relatively high at this time point. As discussed in relation to IL1 $\beta$  expression this may be an endogenous analgesic response, perhaps, if these inflammatory mediators remained elevated, hyperalgesia and allodynia would be more intense than reported levels. The high levels of CXCL1 expression in the front of the brain suggest a role for this chemokine in descending modulation.

At 24 hours, expression of CXCL1 mRNA in the middle brain regions was significantly higher than in front and back regions. It is possible that, at this time point, CXCL1 is mediating activity within the limbic system and memory centres where it is contributing to alterations in the motivational-effective pain response. Indeed the fact that this chemokine is generally expressed at higher levels in these brain regions implies that it may play a key role in these processes under normal conditions.

#### 6.4.5 CX3CL1 and CX3CR1 Expression

This is the first evidence of alterations in the expression of both fractalkine/CX3CL1 and its receptor, CX3CR1, in the brain using a pain model. Previously both CX3CL1 and CX3CR1 have been shown to be upregulated in the DRG of the spinal cord in neuropathic pain models (Milligan *et al.*, 2005, Han and Neugebauer, 2005, Axen and Porath, 1966) and a rat model of mono-arthritis (Sun *et al.*, 2007). Here both receptor and ligand were significantly down-regulated in the back regions of the brain at 6, 24 and 72 hours post-injection. There are several possible explanations for this, firstly fractalkine exists on the neuronal cell membrane and is released following neuronal activation, if such release is occurring in the back of the brain due to nociceptive stimulation this may cause a decrease in mRNA expression of the ligand and receptor due to negative feedback from high levels of circulating fractalkine. CX3CL1 expression remains significantly lower in the back of the brain at 24 and 72 hours before gradually returning to baseline, this may be due to reduced neuronal activity at these time points decreasing negative feedback at 7 and 14 weeks or as a result of the increase in IL1 $\beta$  and TNF $\alpha$ .

## **Chapter 7**

# **7 Experimental: Expression of GFAP and Hypoxia in Rat Brain.**

## **7.1 Introduction and Aims**

### **7.1.1 Introduction**

Activation of the glial cells, microglia and astrocytes, within the CNS has been demonstrated to play a role in the development and maintenance of pain. Glia are activated in response to inflammatory mediators and then release further inflammatory molecules along with other mediators of neuronal activity. The astrocyte marker glial fibrillary acid protein (GFAP) is upregulated in pain and has been suggested to mediate the mechanisms related to chronic pain. Building on the results of Chapter 6, where altered expression of the mRNA of multiple inflammatory chemokines and cytokines was demonstrated in the brain during peripheral inflammation, the expression of this marker was measured in the brains of CFA footpad treated animals.

As previously discussed, hypoxia and inflammation are closely related; hypoxia can lead to inflammation, for example in stroke or following damage to a blood vessel. Ischaemia reperfusion models in animals have provided evidence of this in both the CNS and the periphery. In turn, inflammation has also been demonstrated to cause hypoxia, tissue swelling can cause vasoconstriction and release of inflammatory mediators

### **7.1.2 Aims**

To further investigate the inflammatory response in the rat brain in the inflammatory footpad model by examining the profile of GFAP expression at acute, sub-acute and chronic time points.



To investigate the hypothesis that hypoxia may be present in the CNS in chronic pain, it was decided to use Hypoxyprobe (see 3.4.2) to investigate potential hypoxia in the rat brain in the inflammatory footpad model at acute, sub-acute, and chronic time points.

## **7.2 Materials and Methods**

### **7.2.1 Blocking and Cutting**

#### **7.2.1.1 Materials**

Superfrost slides, and isopentane, (Fisher Scientific, Leics, UK), 2mm rat. Coronal brain matrix (Kent Scientific Corporation, Torrington, Connecticut, USA). Optimum cutting temperature compound (OCT). Cork disks and razor blades (RA Lamb Ltd, East Sussex, UK). Liquid Nitrogen (BOC, Surrey, UK)

#### **7.2.1.2 Methods**

Brains from experiments described in section 5.3.2 were removed from storage at -70°C and warmed to -20°C for use as follows; To prepare brains for cutting they were cut into 2mm blocks and mounted on cork disks in optimal cutting temperature compound (OCT) in freezing/melting isopentane. A brain matrix was used to cut 2mm blocks and enable consistent and precise cutting of sections. Blocks were stored in 50ml centrifuge tubes at -70°C until cut for sections.

Section 6 from the middle of the brain was chosen for analysis since this brain region contains many regions important in pain regulation including, the hippocampus, the thalamus and the area of the somatosensory cortex that represents the hind paw.

16µm sections for staining were cut by cryostat at -15°C and then mounted on superfrost slides. Slides were left to air dry overnight at room temperature and stored at -20°C until used in staining protocols.

## **7.2.2 Staining for Hypoxyprobe, and GFAP**

### **7.2.2.1 Materials**

PBS, acetone, coverslips and PAP pen (Fisher Scientific, Leics, UK). Rabbit polyclonal anti-Hypoxyprobe (PAb 2627), (HPI Inc., Burlington, MA, USA). Rabbit anti-GFAP (ab7779) (Abcam Ltd., Cambridge, UK). Vectastain ABC kit (alkaline phosphatase rabbit IgG), (Vector Laboratories Ltd, Orton Southgate, Peterborough, UK). Mayer's Haematoxylin, Fast red TR/Naphthol tablets, (Sigma-Aldrich Co Ltd Poole, UK). Aquamount (VWR International, Poole, UK)

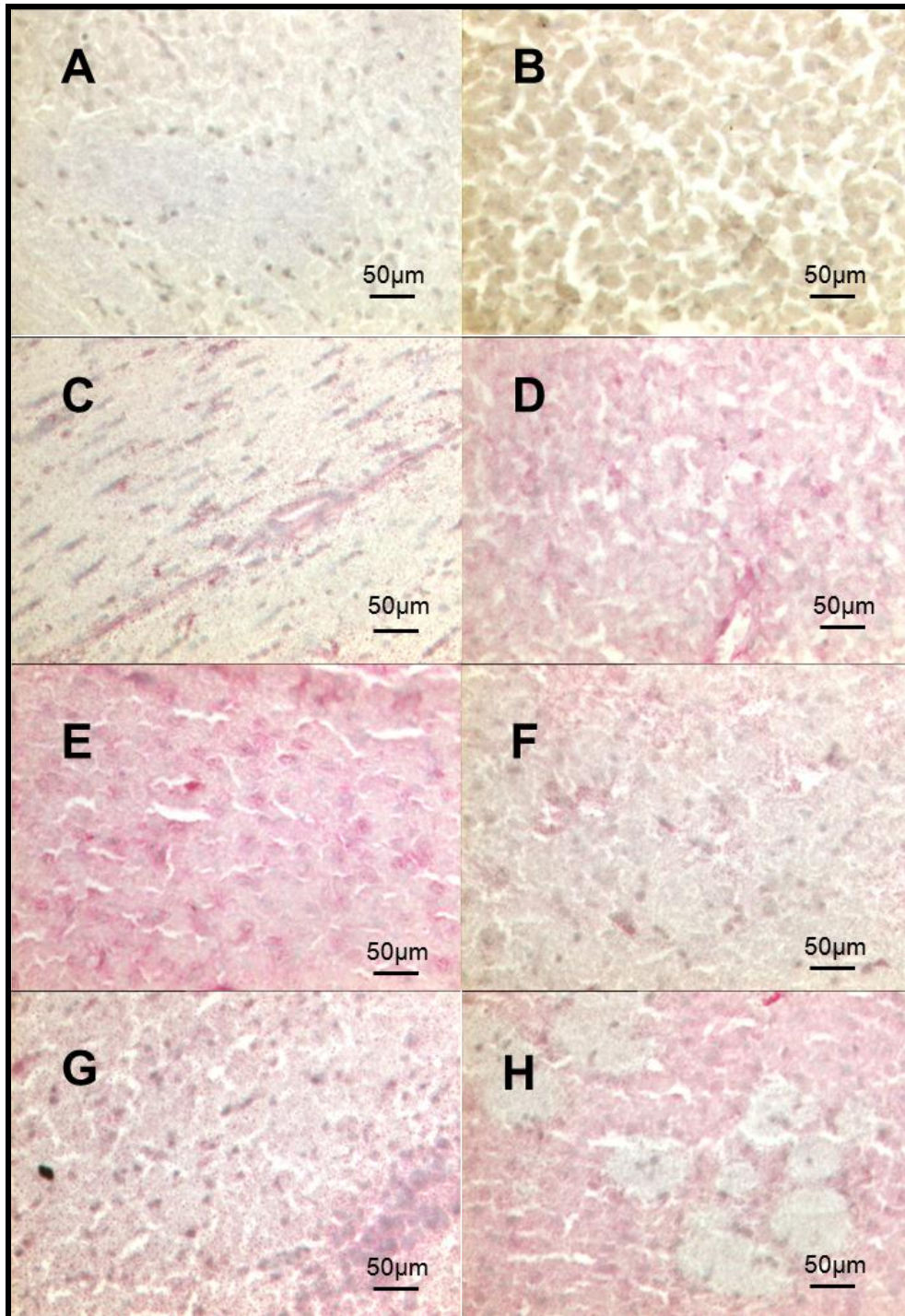
### **7.2.2.2 Methods**

Slides prepared as in section 7.2.1 were removed from the freezer and warmed to room temperature, tissue was then fixed for ten minutes in acetone at 4°C. Following fixation excess acetone was evaporated from the slides and a PAP pen used to provide a hydrophobic barrier around the sections. The remainder of the procedure was carried out according to the protocol for the Vectastain ABC kit. Briefly, sections were washed three times for five minutes in PBS. Following washing, in order to prevent irrelevant binding of antibodies, tissue was blocked for one hour at room temperature using a blocking solution of 15% serum in PBS. Blocking solution was decanted and sections were incubated overnight in a humidified chamber at 4°C with primary antibody diluted in blocking solution. Antibody concentrations varied, see results section for further details. In each experiment two negative controls were also used, a no primary control, where sections were incubated with blocking solution only (see figure 38 A) and a serum control, where sections were incubated with serum from the primary antibody host species diluted in blocking solution (see figure 38 B). Following overnight incubation the primary antibody solution was decanted and sections washed three times for five minutes in PBS. Sections were then incubated at room temperature for one hour with a 1:200 solution of biotinylated secondary antibody, washed three times for five minutes in PBS

and then incubated for thirty minutes in ABC-AP solution before washing for five minutes in PBS. Slides were then incubated in alkaline phosphatase solution (Fast Red TR/Naphthol made up according to the manufacturer's instructions) for 5-20 minutes depending on the antigen of interest and the reaction terminated in tap water. Tissue was counterstained for five minutes in Mayer's haematoxylin and washed in swirling tap water for ten minutes prior to mounting in Aquamount.

### **7.2.3 Analysis of slides**

Slides were graded according to the level and intensity of staining and the experimenter was blinded to the treatment group during the analysis. The grading protocol was based on that of Colburn *et al* (1997) where each section was awarded a score according to the degree of staining, 0 being no staining, 1 mild staining, 2 moderate and 3 intense (see Figure 38). Four sections were scored from each brain, meaning a total of sixteen for each experimental group and scoring was blinded and randomised.

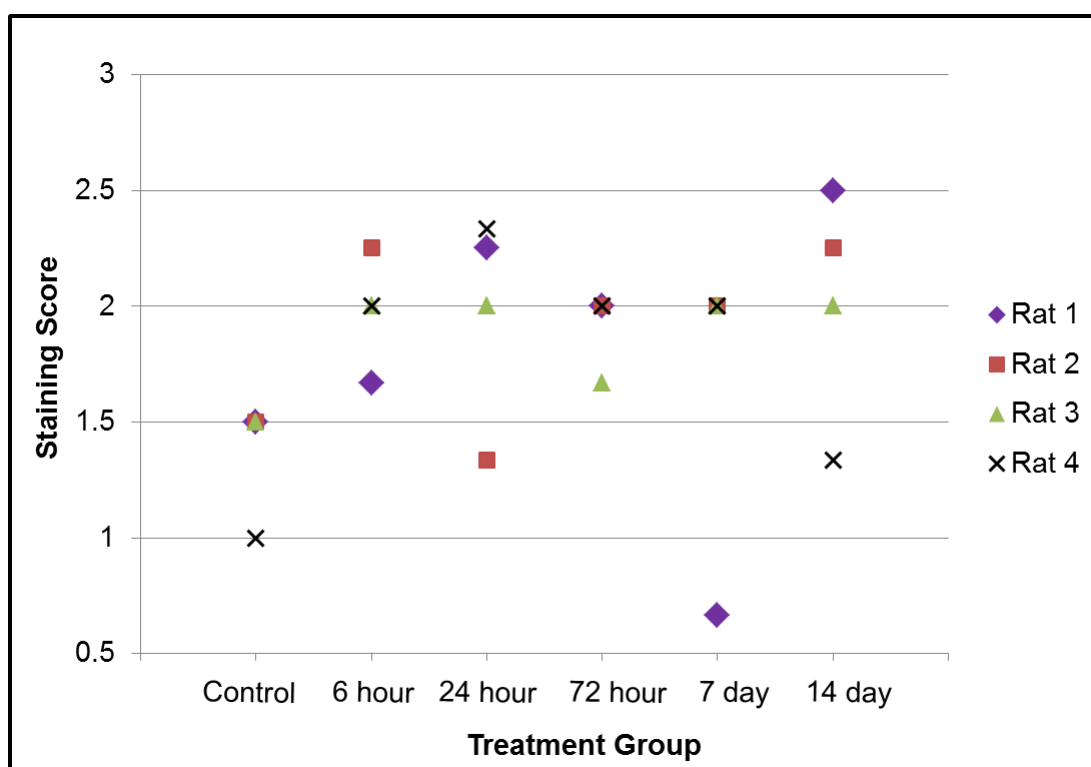


**Figure 38 Representative micrographs of hypoxyprobe and GFAP staining X200** A) Control, no primary antibody. B) Serum control. C) GFAP staining scored 1 D) GFAP staining scored 2 E) GFAP staining scored 3 F) Hypoxyprobe staining scored 1 G) Hypoxyprobe staining scored 2 H) Hypoxyprobe staining scored 3

## 7.3 Results

### 7.3.1 GFAP Staining

Baseline GFAP staining representative of inactivated astrocytes is present in all treatment groups. Higher levels of staining was seen in the brains of all treatment groups with three out of four animals in each group showing a staining score of two or more (see figure 39 and table 6). Highest levels of staining were at the 6 and 24 hour and 14 day timepoints which correlates with the highest levels of paw oedema described previously (see figure 24).

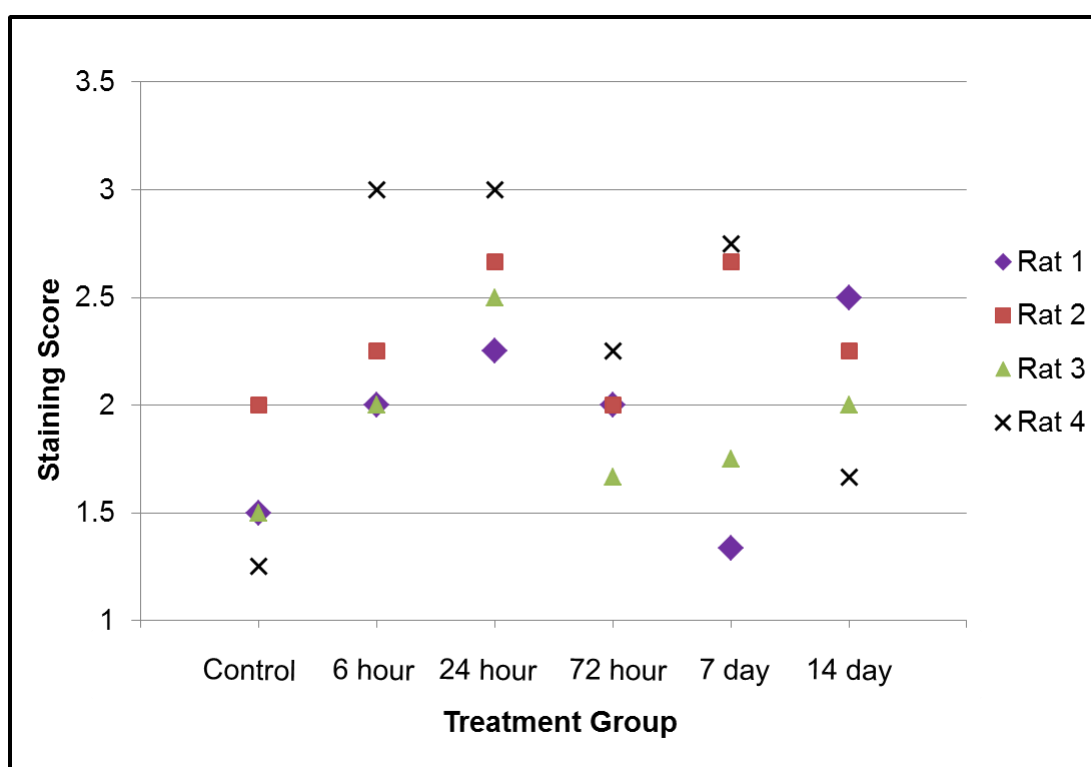


**Figure 39 Scatter diagram of mean GFAP staining score for each rat in the right brain of CFA treated rats and non-injected controls.** Expression is increased at all time points post injection. n =4 for each animal unless otherwise indicated (see table 6).

Treatment Group	Rat 1	Rat 2	Rat 3	Rat 4
Normal	2.00	x	1.00	0.00
	1.00	1.00	1.00	2.00
	1.00	x	2.00	1.00
	2.00	2.00	2.00	1.00
Mean	1.50	1.50	1.50	1.00
6 hour CFA	2.00	2.00	2.00	2.00
	x	2.00	2.00	2.00
	1.00	3.00	2.00	2.00
	2.00	2.00	2.00	2.00
Mean	1.67	2.25	2.00	2.00
24 hour CFA	2.00	2.00	2.00	3.00
	2.00	2.00	2.00	2.00
	3.00	x	2.00	x
	2.00	0.00	2.00	2.00
Mean	2.25	1.33	2.00	2.33
72 hour CFA	x	2.00	2.00	2.00
	2.00	2.00	2.00	2.00
	2.00	2.00	1.00	2.00
	2.00	2.00	x	2.00
Mean	2.00	2.00	1.67	2.00
7 day CFA	x	2.00	2.00	2.00
	2.00	2.00	2.00	2.00
	0.00	x	2.00	2.00
	0.00	2.00	2.00	2.00
Mean	0.67	2.00	2.00	2.00
14 day CFA	2.00	3.00	2.00	2.00
	3.00	2.00	2.00	x
	3.00	2.00	x	1.00
	2.00	2.00	2.00	1.00
Mean	2.50	2.25	2.00	1.33

**Table 6 Individual GFAP staining scores for right (ipsilateral) brain sections.** Sections were scored 0-3 according to intensity of staining (see figure 38), x unable to score section.

A similar pattern of staining was seen in the left side of the brain, contralateral to the injected paw. Baseline staining with a score of 1-2 was seen in all control animals with the highest scores seen at 6 hours, where all sections scored 2 or above and 24 hours, where three out four sections scored 2.5-3 (see figure 40 and table 7). These increases correspond with the increase in paw swelling described in Chapter 5, interestingly at 72 hours, where there is a decrease in paw volume, there is a concurrent decrease in GFAP expression. Later timepoints had higher than baseline staining, with an increase at 7 and 14 days compared with 72 hours again correlating with the increases seen in paw volume.



**Figure 40 Scatter diagram of mean GFAP staining score for each rat in the left brain of CFA treated rats and non-injected controls.** Expression is increased at all time points post injection. n = 4 for each animal unless otherwise indicated (see table 7).

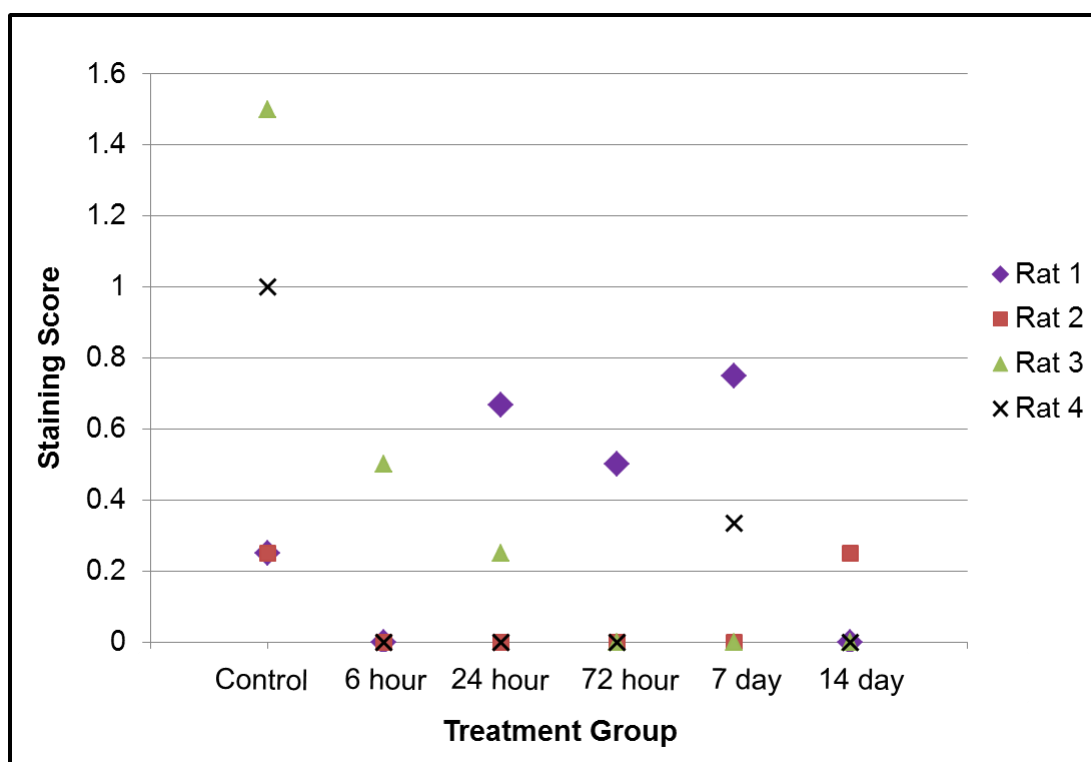
Treatment Group	Rat 1	Rat 2	Rat 3	Rat 4
Normal	2.0	x	1.0	1.0
	1.0	1.0	1.0	2.0
	1.0	x	2.0	1.0
	2.0	3.0	2.0	1.0
Mean	1.5	2.0	1.5	1.3
6 hour CFA	2.0	2.0	2.0	3.0
	2.0	2.0	2.0	3.0
	x	3.0	2.0	3.0
	2.0	2.0	2.0	3.0
Mean	2.0	2.3	2.0	3.0
24 hour CFA	2.0	3.0	3.0	3.0
	2.0	3.0	2.0	3.0
	3.0	x	2.0	x
	2.0	2.0	3.0	3.0
Mean	2.3	2.7	2.5	3.0
72 hour CFA	x	2.0	2.0	2.0
	2.0	2.0	2.0	2.0
	2.0	2.0	1.0	2.0
	2.0	2.0	x	3.0
Mean	2.0	2.0	1.7	2.3
7 day CFA	x	3.0	2.0	3.0
	2.0	2.0	1.0	3.0
	0.0	x	2.0	3.0
	2.0	3.0	2.0	2.0
Mean	1.3	2.7	1.8	2.8
14 day CFA	2.0	3.0	2.0	2.0
	3.0	2.0	2.0	x
	3.0	2.0	2.0	2.0
	2.0	2.0	2.0	1.0
Mean	2.5	2.3	2.0	1.7

**Table 7 Individual GFAP staining scores for left (contralateral) brain sections.** Sections were scored 0-3 according to intensity of staining (see figure 38), x unable to score section.



### 7.3.2 Hypoxyprobe Staining

In the right side of the brain the highest level of Hypoxyprobe staining was seen in non-injected animals although scores here still remained low with only 2 rats scoring 1 or above (see figure 41 and table 8). At post-injection time-points the all sections were scored 1 or below, with most samples expressing little or no staining for Hypoxyprobe.

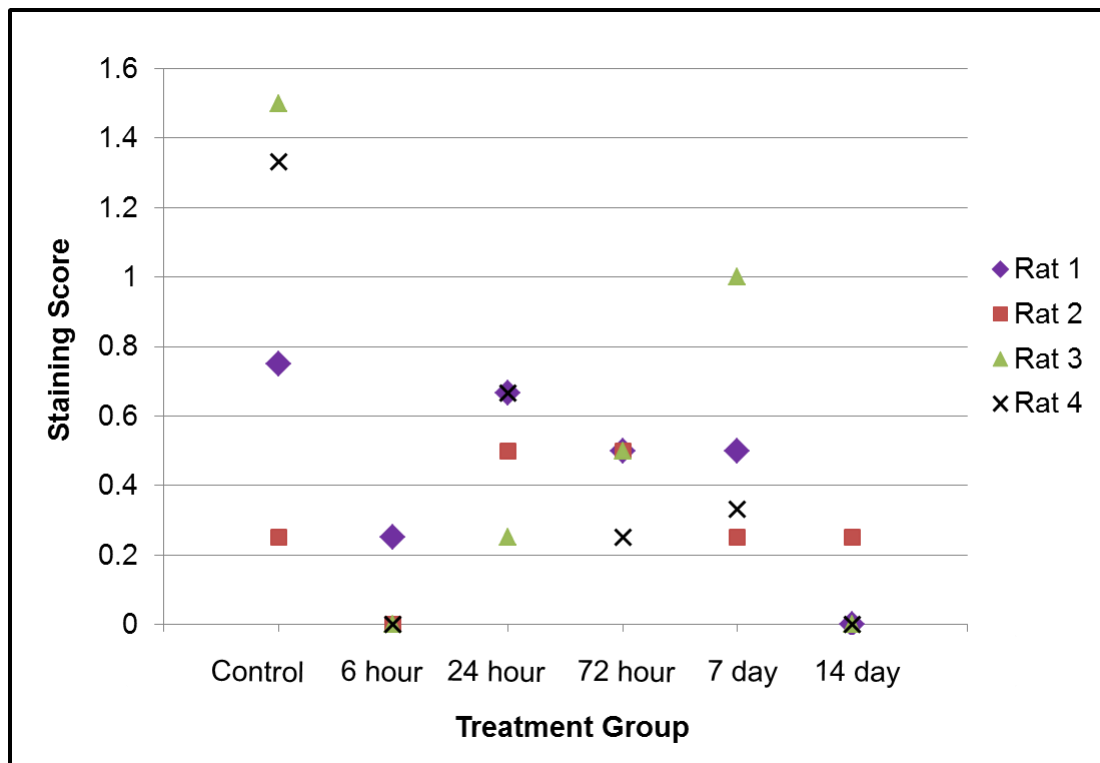


**Figure 41 Scatter diagram of mean Hypoxyprobe staining score for each rat in the right brain of CFA treated rats and non-injected controls. n = 4 for each animal unless otherwise indicated (see table 8).**

Treatment Group	Rat 1	Rat 2	Rat 3	Rat 4
Normal	0.0	1.0	2.0	1.0
	0.0	0.0	1.0	1.0
	1.0	0.0	2.0	x
	0.0	0.0	1.0	1.0
Mean	0.3	0.3	1.5	1.0
6 hour CFA	0.0	0.0	1.0	x
	0.0	0.0	1.0	0.0
	0.0	0.0	0.0	0.0
	0.0	0.0	0.0	0.0
Mean	0.0	0.0	0.5	0.0
24 hour CFA	x	0.0	0.0	0.0
	0.0	0.0	0.0	0.0
	1.0	0.0	1.0	0.0
	1.0	0.0	0.0	x
Mean	0.7	0.0	0.3	0.0
72 hour CFA	0.0	0.0	0.0	0.0
	0.0	0.0	0.0	0.0
	1.0	0.0	0.0	0.0
	1.0	0.0	0.0	0.0
Mean	0.5	0.0	0.0	0.0
7 day CFA	1.0	0.0	0.0	1.0
	1.0	0.0	0.0	0.0
	0.0	0.0	0.0	x
	1.0	0.0	0.0	0.0
Mean	0.8	0.0	0.0	0.3
14 day CFA	0.0	0.0	0.0	0.0
	0.0	1.0	0.0	0.0
	0.0	0.0	0.0	0.0
	0.0	0.0	0.0	0.0
Mean	0.0	0.3	0.0	0.0

**Table 8 Individual Hypoxyprobe staining scores for right (ipsilateral) brain sections.** Sections were scored 0-3 according to intensity of staining (see figure 38), x unable to score section

A similar pattern of Hypoxyprobe staining was seen in the left side of the brain, contralateral to the injected paw with two of four control animals having a moderate level of staining and all remaining groups showing little or no staining (see figure 42 and table 9). These data showed a higher variance than that seen in the right side of the brain with several more sections from injected animals showing some baseline staining.



**Figure 42** Scatter diagram of mean Hypoxyprobe staining score for each rat in the left brain of CFA treated rats and non-injected controls. n = 4 for each animal unless otherwise indicated (see table 9).

Treatment Group	Rat 1	Rat 2	Rat 3	Rat 4
Normal	1.0	1.0	2.0	2.0
	0.0	0.0	1.0	1.0
	1.0	0.0	2.0	x
	1.0	0.0	1.0	1.0
Mean	0.8	0.3	1.5	1.3
6 hour CFA	0.0	0.0	0.0	x
	1.0	0.0	0.0	0.0
	0.0	0.0	0.0	0.0
	0.0	0.0	0.0	0.0
Mean	0.3	0.0	0.0	0.0
24 hour CFA	x	1.0	1.0	1.0
	0.0	1.0	0.0	1.0
	1.0	0.0	0.0	0.0
	1.0	0.0	0.0	x
Mean	0.7	0.5	0.3	0.7
72 hour CFA	0.0	0.0	1.0	1.0
	0.0	0.0	0.0	0.0
	1.0	1.0	0.0	0.0
	1.0	1.0	1.0	0.0
Mean	0.5	0.5	0.5	0.3
7 day CFA	0.0	1.0	1.0	1.0
	0.0	0.0	1.0	0.0
	1.0	0.0	1.0	x
	1.0	0.0	1.0	0.0
Mean	0.5	0.3	1.0	0.3
14 day CFA	0.0	0.0	0.0	0.0
	0.0	1.0	0.0	0.0
	0.0	0.0	0.0	0.0
	0.0	0.0	0.0	0.0
Mean	0.0	0.3	0.0	0.0

**Table 9 Individual Hypoxyprobe staining scores for left (contralateral) brain sections.** Sections were scored 0-1 according to intensity of staining (see figure 38), x unable to score section.

## 7.4 Discussion

### 7.4.1 GFAP Expression

The results of GFAP staining are concurrent with those of Chapters 6 and 7, increased staining was seen at 6 and 24 hours and 7 and 14 days post-injection, corresponding with an increase in paw swelling and release of inflammatory mediators that has been described at these time points. Interestingly, these increases in activation were seen in both sides of the brain and not just in the left side, contralateral to the injected paw. The increase in GFAP expression in the right side of the brain, particularly at later time points, corresponds to the development of hyperalgesia in the left paw described by Raghavendra *et al* (2004), no increase in swelling was evident in the left paw of injected rats at this time point but given the evidence it seems likely that an increase in hyperalgesia and allodynia would have developed which would be followed by an increase in paw volume. This supports the hypothesis that glial cell cross talk within the brain contributes to the development of referred pain. If inflammatory changes can contribute to these sensory changes it is likely that alterations in brain morphology may also be contributing to brain changes that lead to the development of the motivational-effective symptoms of chronic pain.

It is interesting that some differences were seen between the left and right sides of the brain here when no difference was observed in Chapter 6, there are several possible explanations for this; Firstly, in these immunohistochemical studies a thin section from the middle of the brain was used meaning a very specific area of the brain was studied. In the PCR experiments of Chapter 6 a larger region was homogenised and finer changes may have been lost. Together these results suggest that throughout the mid-brain regions there are variations in expression of inflammatory markers on both the ipsilateral and contralateral side to the peripheral insult. Secondly in this study the activation of astrocytes was measured through the expression of GFAP, in Chapter 6 mRNA expression was measured, it is possible that mRNA for inflammatory mediators is produced on both sides of

the brain in response to painful inflammation in the periphery but transcribed and released differentially depending on sensory input and glial activation. Finally, as discussed in 2.2.1, astrocytes are not the only cells involved in the immune response within the CNS, the microglia are also known to play a key role and may be differentially activated to the astrocytes in this model.

#### **7.4.2 Hypoxyprobe Staining**

The results of the Hypoxyprobe immunohistochemistry were largely unexpected with the highest levels of staining observed in the non-injected controls. An explanation for this may be that a mild state of hypoxia is induced in the tissues following termination prior to perseveration by freezing, which remains evident in non-injected controls. Inflammation can be associated with the release of reactive oxygen species (ROS) which can lead to oxidative damage, a process which has been demonstrated to play a role in neurodegenerative diseases such as Alzheimer's and Parkinson's. One hypothesis is that ROS are released in the brain during the acute and sub-acute stages of this pain model reversing the hypoxia that is seen in non-injected animals. At later stages hypoxia may be seen due to swelling or tissue damage within the brain as a result of the longer term inflammatory insult.

#### **7.4.3 Conclusions**

The results presented in this chapter warrant further investigation. The evidence provided here suggests a key role for GFAP in the response to inflammatory pain which may vary in different brain areas. The Hypoxyprobe results, although slightly inconsistent, indicate that there is variation in oxygenation levels within areas of the brain in persistent pain and a role for ROS within the CNS in chronic pain has been suggested.

Due to difficulties with staining it was not possible to do in depth statistical analysis using these tissues nor was it possible to consistently locate specific nuclei within brain regions. It is likely that further experiments would benefit

from using perfused wax embedded tissue, a method that would allow for finer sections to be cut leading to a clearer result suitable for image analysis.

## **Chapter 8**

# **8 Methodology: The Development of a Primary Cell Culture Protocol**

## **8.1 Introduction**

In this chapter a method for the culture and characterisation of primary cell lines was developed to be used in future experiments. Due to time constraints it was not possible to use the protocol further, the development of the method is described here.

## **8.2 Materials and Methods**

### **8.2.1 Materials**

Glutamine, poly-L-lysine solution and poly-L-lysine hydrobromide (Sigma-Aldrich CO Ltd., Poole, UK). Sterile distilled water,  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  free HBSS, 10mM HEPES (pH 7.4) Neurobasal, B27 supplement, trypan blue, trypsin 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen Ltd, Paisley. UK).

### **8.2.2 Optimisation of primary cell culture**

The following method is based on that set out by Banker and Goslin (Banker & Goslin 1998) and was developed over a series of pilot experiments.

#### **8.2.2.1 Coverslip preparation**

To enable the cells to be used in further experiments such as in immunohistochemistry they are grown on coverslips within six well plates. This allows easy treatment and maintenance and also allows for simple transfer of the cells for further experimentation.



In order for isolated cells to grow *in vitro* they require a substrate to grow on to allow them to attach, for neurons poly-L-lysine is the most commonly used. Initial experiments were done using poly-L-lysine hydrobromide in 1mg/ml solution of distilled water which was then filter sterilised. Although this produced some satisfactory results making up solutions was time consuming and the risk of contamination quite high. It was therefore decided to do future work with a pre-prepared and sterilised solution (Sigma), this enabled greater consistency and ease of use across experiments and the solution is more stable and easier to store than poly-L-lysine hydrobromide.

Coverslips (22mm<sup>2</sup>) were sterilised by autoclave and then placed in six well plates. Slips were then coated with poly-L-lysine solution (1mg/ml, Sigma), approximately 0.5ml was added to each well and the plate rocked to evenly cover each slip. In order to allow the solution to adhere to the slips, plates were then incubated at 37°C overnight. The following day any excess poly-L-lysine was removed by aspiration and plates were washed three times for 30 minutes each wash in sterile distilled water (Gibco/Invitrogen), this step is essential as excess poly-L-lysine can prove toxic to the cells. If plates were not used immediately they were kept under sterile water at 2-8°C until needed.

According to the original method of Banker and Goslin (Banker & Goslin 1998) the ideal media for culturing neurons is Eagles minimum essential medium with hormones and N<sub>2</sub> supplements added. The cells then had to be plated with an astrocyte 'feeder layer' to provide some of the essential growth factors they needed. Over recent years media and supplements have been developed specifically for the growth of primary cultures of embryonic neurons (Brewer *et al.* 1993; Brewer 1995) which remove the need for a feeder layer. In this work Neurobasal (Invitrogen) medium was used supplemented with the B27 serum free supplement (Invitrogen) containing growth factors and nutrients essential for neuronal growth and development. The media was also supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen) to help prevent infection and 2mM L-Glutamine as

this essential amino acid which is unstable in media and breaks down in media kept over long time periods.

On the day of isolation water was removed from the plates by aspiration and 4ml of plating media (as described above) added to each well. Plates were then incubated at 37°C/5% CO<sub>2</sub> until needed (for at least 30 mins), this incubation is essential to allow the media to stabilise at the correct pH and temperature to maintain the cells.

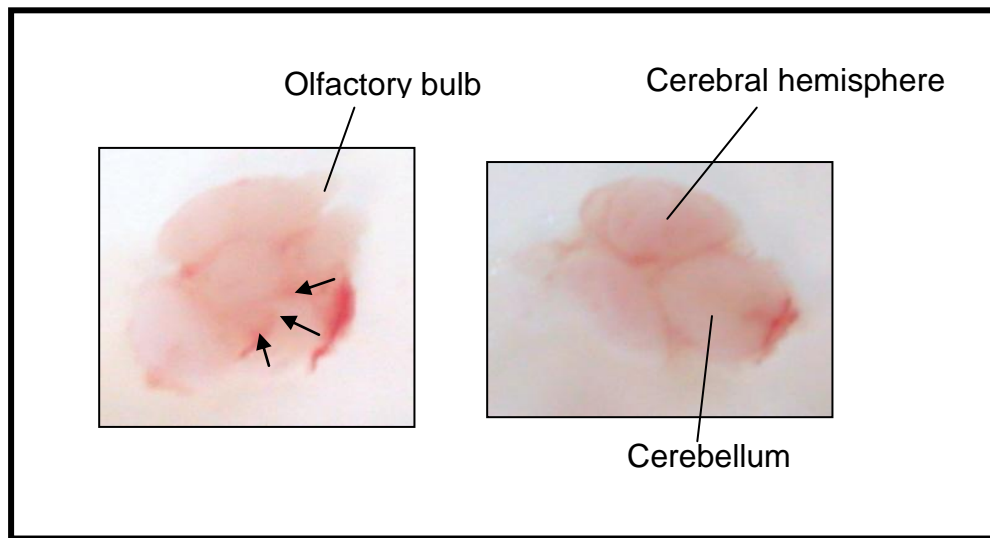
#### **8.2.2.2 Cell isolation and plating**

All animals used were from the University of Bath breeding colonies and were kept in the animal house with a twelve hour light dark cycle and free access to food and water.

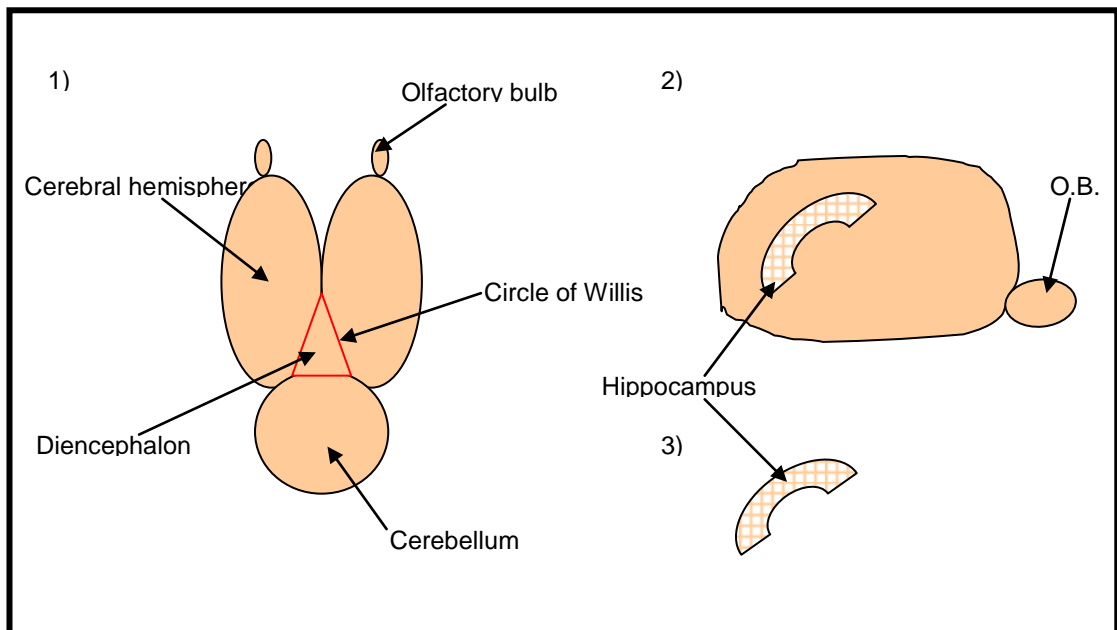
Neuronal culture can be done using cells from adult rats (Brewer 1997) however this is a more difficult and time consuming procedure with inconsistent results. Neurons in an adult brain have formed a complex matrix with many connections to one another and other cells within the brain. Disturbing this matrix by dissection causes damage to the delicate cells and disrupts extracellular connections therefore, most culture of hippocampal neurons is done using foetal cells. Between 17 and 21 days gestation (E17-E21) has been demonstrated to produce optimum results (Banker & Goslin 1998) and initial experiments here were done at E18. The dissection proved difficult with rats of this age and it was decided to use rats at E19 in future work.

A timed pregnant Sprague Dawley rat (E19) was euthanised by CO<sub>2</sub>. Foetuses were removed by caesarean section and decapitated, heads were then placed in sterile Ca<sup>2+</sup> and Mg<sup>2+</sup> free HBSS supplemented with 10mM HEPES (pH 7.4) and the brains removed. Brains were placed in a sterile Petri dish with fresh HBSS/HEPES and kept on ice (see figure 43). The buffer prevents damage to the cells by osmotic and pH challenge, it is

essential that this part of the protocol is completed as fast as possible to prevent degradation of the tissues.



**Figure 43** An E19 foetal rat brain the ventral view on the left and the dorsal on the right. The arrows indicate the circle of Willis, a formation of blood vessels that separates each of the cerebral hemispheres from the diencephalon.



**Figure 44 Schematic representation of the dissection process.** 1) A ventral view of the brain, the cerebral hemispheres are removed by cutting along the circle of Willis and down the midline. 2) The location of the hippocampus within the left hemisphere viewed from the midline. Following careful removal of the meninges the hippocampus is dissected out using Dumont forceps. 3) The free hippocampus.

Using a dissecting microscope hippocampi were dissected out and placed in sterile HBSS/HEPES and again kept on ice (see figure 44).

Once all the hippocampi had been removed, they were transferred to a centrifuge tube and the volume made up to 4.75ml with HBSS/HEPES.

Trypsin (250µl 5% Invitrogen) was added and hippocampi incubated at 37°C for 10 mins. This allows for enzymatic degradation of the tissue into individual cells. Incubating the cells for too long can cause damage and too little time causes the cells to grow in clumps where it is impossible for them to develop properly (see figure 45). Following incubation the trypsin/buffer solution was removed and the hippocampi were washed twice with HBSS/HEPES and once with plating medium (5 minutes each wash) to allow all trypsin to diffuse from the tissue and prevent further degradation.



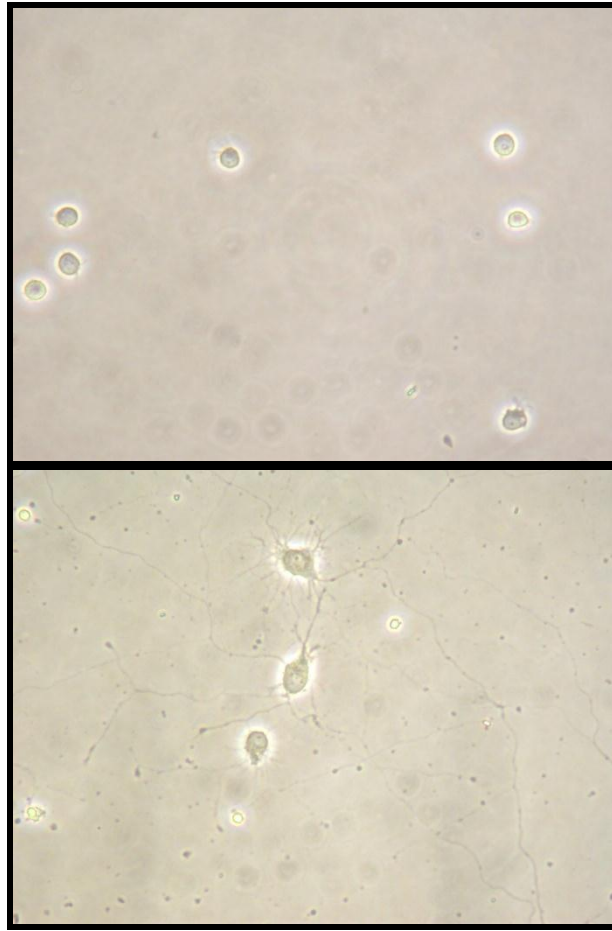
**Figure 45 Cells 7 days in culture after inadequate trypsinisation.** Many cells are clumped together extending multiple axons between clumps. The cells in the centre of the clumps will be starved of nutrients and are unable to form axons and dendrites.

The contents of the tube were then made up to ~4ml and triturated using a 1ml Gilson pipette for approximately 2 mins to mechanically break the tissue down into individual cells. Viable cells were counted using an exclusion assay. 10µl of trypan blue (Invitrogen) was added to 10µl of the cell suspension and incubated for 2 mins. Damaged cells absorb the dye and so appear blue, viable cells were then counted using a haemocytometer and approximately 50,000-80,000 cells plated in each well depending on the

experiment. Plates were briefly swirled to evenly distribute cells and then incubated at 37°C 5% CO<sub>2</sub>.

Cell growth was monitored and at approximately day three after plating half of the media was removed from each well and replaced with media supplemented with cytosine arabinoside (to a final concentration of 10µM ara-c) to inhibit glial proliferation. Cells were maintained at 37°C/5% CO<sub>2</sub> for up to one month with half their media replaced once a week.

In order to ensure the accuracy of the isolation and culture protocol the cells must be 'characterised', this involves looking at the cell structure and general morphology along with identification of certain key proteins or products. In this case pyramidal cells are fairly easy to identify by their specific shape of a pyramidal shaped body with a single axon and long branching dendrite (see figure 4 (bottom) and figure 47). Cells were also stained with neuron specific antibodies, the development of the protocol used is described in the following section.



**Figure 46 Individual cells immediately after plating (top) and after five days in culture (bottom).**

### **8.3 Optimisation of Immunocytochemistry**

Immunohistochemistry is an excellent tool for identifying and characterising cell lines. Here the aim was to characterise the cells from primary rat hippocampal cultures, as both glial and neuronal cells are found in the hippocampus and it is important to know the ratio of each in the culture. Characterisation of the cells also serves as a check of the accuracy of the dissection process and the success of the culture procedure in general.

Selection of antibodies is key to ensure that they are specific to the individual protein and that they are the correct antibody for the method. When using multiple antibodies for double or triple staining it is important that they have all been raised in different species so as to avoid cross-reactivity. The

antibodies used here were all supplied by Abcam and their specificity and the rationale for using them is described below:

### **8.3.1 Materials: Primary antibodies**

#### **8.3.1.1 Glial fibrillary acidic protein (GFAP)**

GFAP is a component of glial cells, it was identified as being specific to these immunological cells and is not found in neurons. It is expressed in astrocytes in brain tissue and in the Schwann, satellite and enteric glial cells of the peripheral nervous system. Here anti-GFAP is used to identify the purity of the culture since for most experiments only neurons are required and to identify the best method for achieving pure cultures.

#### **8.3.1.2 Growth associated protein 43 (GAP43)**

As its name suggests, GAP43 is expressed in growing and regenerating neurons it is expressed within the axonal cytoplasmic surface and the growth cones of extending axons. It is sometimes also expressed in reactive glial cells. As such its expression is greater during early stages of neuronal development and following damage. The aim here is to use it to characterise neurons initially and in future experiments it may prove useful when looking at growth and development of neurons following treatment.

#### **8.3.1.3 Microtubule associated protein 2 (MAP2)**

MAP2 is the major microtubule associated protein of brain tissue. There are three forms of MAP2 (MAP2a MAP2b and MAP2c), MAP2b and MAP2c are present in the newborn rat brain but MAP2a does not appear until between postnatal days 10 and 20. At the same time, the level of MAP2c drops by 10-fold, this change happens during the period when dendrite growth is completed and when neurons have reached their mature morphology. MAP2 is known to promote microtubule assembly and to form side-arms on microtubules. It also interacts with neurofilaments, actin, and other elements

of the cytoskeleton. The antibody chosen is known to react with both MAP2a and MAP2b making it a good indicator of neuronal differentiation.

### **8.3.2 Materials: Secondary antibodies**

Two secondaries were used since both GFAP and GAP3 were raised in rabbit and all double staining was done with the MAP2 antibody (raised in mouse) and one or other of these. The antibodies were conjugated to fluorescent proteins Rhodamine and FITC. Rhodamine fluoresces red when excited at 546nm and FITC green at 450nm.

### **8.3.3 Development of protocol**

In order to obtain the best results it is necessary to identify the ideal antibody concentration for any particular cell line and technique. This is done by using a gradient of both the primary and secondary antibody concentrations and identifying the best end result. Too low a concentration and the protein will not be picked up at all and too high a concentration can lead to excessive background and nonspecific staining making 'true' results difficult to identify.

As well as this other factors such as the fixation, permeabilisation and blocking steps all need to be carefully selected to optimise results. It is important that these steps do not destroy or lose the protein of interest, membrane bound proteins can be lost in permeabilisation and certain fixation methods can be damaging to some proteins. The following protocol is the basic method used, the results obtained with the different antibodies are then described along with the subsequent changes made in the protocol.

### **8.3.4 General protocol for ICC**

#### **8.3.4.1 Fixation**

Media was aspirated and cells rinsed twice with Phosphate buffered saline (PBS). Cells were then fixed by incubation in ice cold 1:1 methanol/acetone



for five minutes. The methanol mixture was aspirated and cells washed twice for five minutes each in ice cold PBS.

#### **8.3.4.2 Permeabilisation**

PBS was aspirated and cells incubated for ten minutes in PBS with 0.5% Triton X-100 (PBST). The PBST was removed and cells washed three times in PBS for five minutes each wash.

#### **8.3.4.3 Blocking and incubation with primary antibodies**

##### Single stain

Cells were incubated with 10% normal serum from the species in which the secondary antibody was raised (goat or sheep) in PBST for thirty minutes at room temperature. The block was then removed and the primary antibody was added made up in blocking solution. Cells were then incubated overnight at 2-4°C.

##### Double stain

Cells were incubated with 1% bovine serum albumin (BSA) in PBST for thirty minutes at room temperature. The block was then removed and a mixture of the two primary antibodies (raised in different species) in blocking solution was added. Cells were incubated overnight at 2-4°C.

#### **8.3.4.4 Secondary antibodies**

The primary antibody solution was decanted and the coverslips washed three times for five minutes in PBS. Cells were then incubated with the secondary antibody (or a mixture of both secondary's for double staining) made up in block for one hour at room temperature, this step must be done in the dark when using fluorescent antibodies. The secondary solution was then removed and the coverslips washed three times for five minutes in PBS (also in the dark if necessary).

#### 8.3.4.5 Counter staining and mounting

Coverslips were mounted onto superfrost slides with vector fluorescence hard mount containing 1.5µg/ml DAPI as a counterstain and viewed under a fluorescent microscope.

#### 8.3.5 Optimisation of antibodies

##### 8.3.5.1 GAP43 and MAP2 single staining

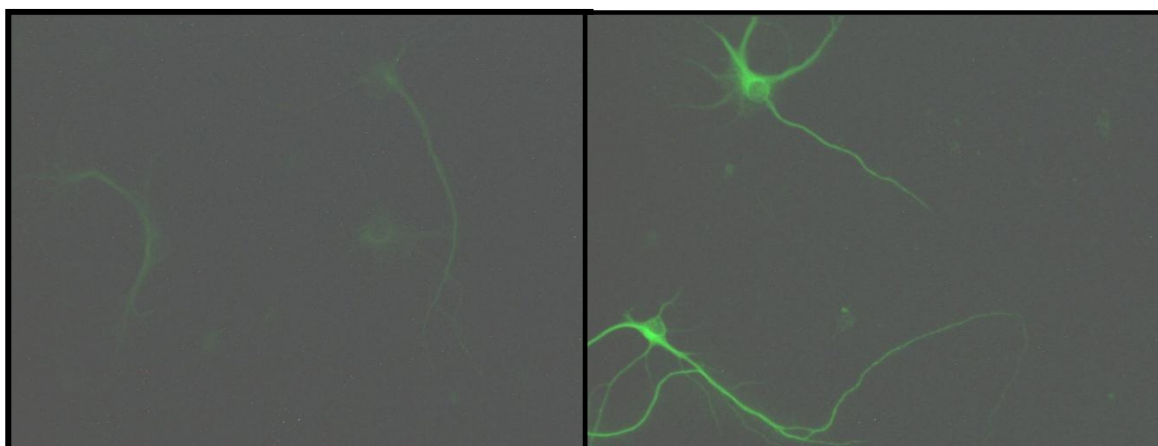
In order to find the ideal concentration of each antibody a range of concentrations was tested based on the concentration suggested by the manufacturer. Cells were stained three weeks after plating. The table below shows the range tested:

Primary	1/200	1/500	1/1000
Secondary	1/200	1/200	1/200
Secondary	1/500	1/500	1/500
Secondary	1/1000	1/1000	1/1000

**Table 10 Antibody concentrations used during antibody optimisation.**

The results of the initial experiment were not ideal. No staining was seen with the GAP43 antibody and MAP2 results were inconsistent so it was not possible to decide on an optimum concentration for each of these antibodies. The experiment was repeated with cells two weeks after plating and the permeabilisation step was omitted as it may have destroyed some of the membrane GAP43 protein.

Again GAP43 staining was unsuccessful, MAP2 was shown to be present in the dendrites, axons and cell bodies of the neurons and it was possible to compare the different concentrations. The clearest stain with the least background was evident with both primary and secondary antibodies at a concentration of 1/200 (see ).

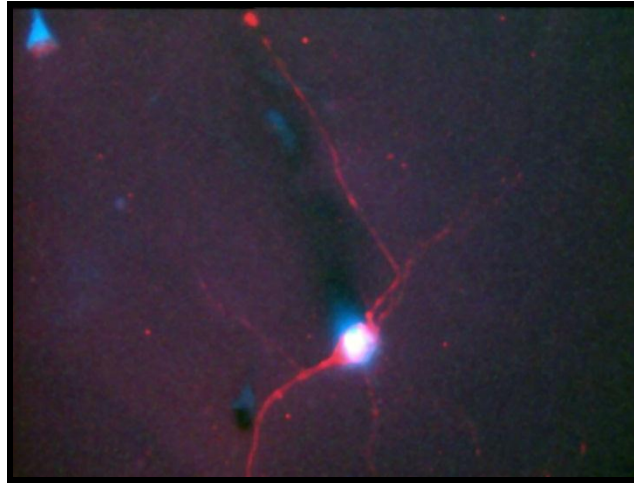


**Figure 47 MAP2 antibody optimisation.** The right hand picture shows the chosen concentration (both antibodies 1/200). The left picture shows an example of less effective staining (primary 1/1000, secondary 1/200).

#### 8.3.5.2 GAP43 single stain

A third attempt to obtain a result with the GAP43 antibody was made, as it is a growth associated protein it was decided to use cells that were only four days in culture. High expression of this protein would be expected in the growth cones of developing axons at this age. The permeabilisation step was again omitted and 100% methanol was used for fixation. Staining was observed in the growth cones and developing axons (see figure 14) and an optimum concentration of 1/1000 for the primary antibody and 1/500 for the secondary was decided on.

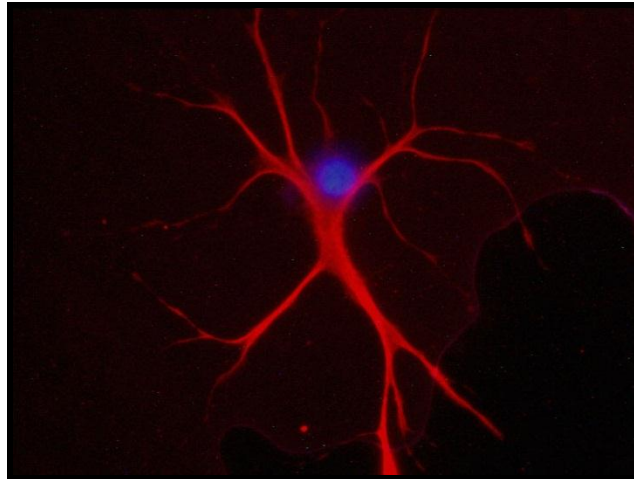
This antibody is not ideal for general characterisation as it seems only to be expressed at certain stages of neuronal development. It may be useful in experiments studying growth, differentiation and recovery of damaged neurons since increased expression would be expected in these conditions.



**Figure 48 GAP43 staining.** An example of GAP43 staining in a cell at four days in culture. There is expression in the cell body and along the axons. The growth cone is brightly stained at the top of the picture.

### 8.3.5.3 GFAP optimisation

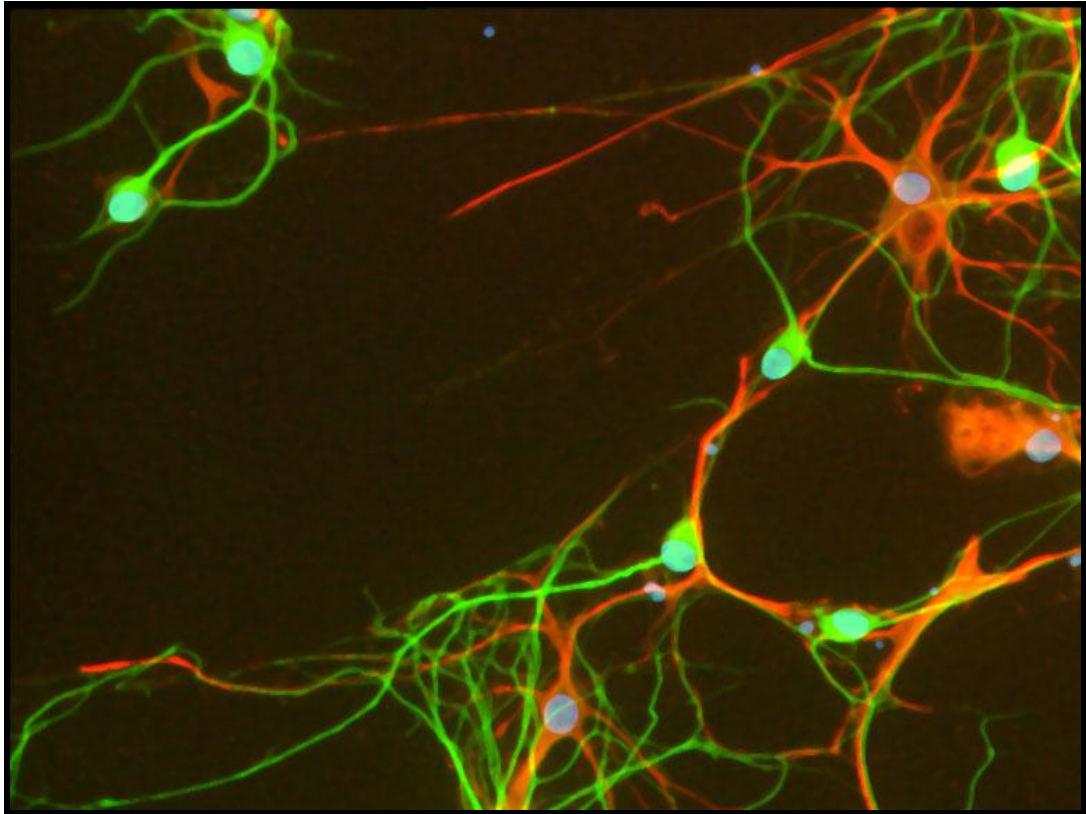
This antibody was also tested at the concentrations listed above. Good results were obtained at all concentrations but at the higher end of the spectrum a large amount of background was seen. It was therefore decided that a concentration of 1/1000 for the primary and 1/500 for the secondary antibodies should be used. Figure 49 shows excellent staining of the cell body and dendrites of a glial cell at these concentrations.



**Figure 49 GFAP staining.** A glial cell stained for GFAP. The nucleus is stained blue with DAPI.

#### **8.3.5.4 Double staining**

Following optimisation of the antibodies for single staining they were used in a double staining protocol. The results of which are shown in Figure 50. The GFAP/MAP2 staining clearly shows the different structure of glial and neuronal cells and there is no evidence of these proteins staining the same cell type. This suggests that these antibodies are excellent tools for the characterisation of these cell types. Again the GAP43 antibody produced inconsistent results.



**Figure 50 Double staining for GFAP and MAP2.** Glial cells are stained red and neurons green. The difference in structure of the two cell types is also clear with the glial cells having a stellate structure and the neurons elongated axons with long branching dendrites.

## 8.4 Discussion

This protocol provides allows for the culture of primary neurons this is beneficial compared with cell lines as such neurons express full functionality and the features that would be seen *in vivo*.

The GFAP and MAP2 antibodies tested here provide excellent markers for glial and neuronal cells respectively. As such they can be used to investigate the purity and viability of hippocampal primary cultures. The GAP43 antibody tested produced inconsistent results, this can in part be attributed to the age of the cultures tested and another double staining experiment needs to be done with 'younger' cells.

## Chapter 9

# 9 General Discussion and Further Work

### 9.1 Discussion

The aim of these studies was to investigate the effect of persistent inflammatory pain on the brain with a specific focus on inflammatory molecules and a potential role for hypoxia. A large body of existing evidence has demonstrated a role for inflammatory mediators within the spinal cord in both neuropathic and inflammatory pain but only a few studies exist which investigate changes within supraspinal regions (Raghavendra *et al.*, 2004, Apkarian *et al.*, 2006).

As discussed in the introductory chapters, chronic pain is associated with many symptoms which cannot be explained by the physical injury itself, indeed, chronic pain can exist without any apparent physical injury. These motivational-affective aspects of pain most significant problems to the researcher as they are often subjective and related to other conditions such as depression, anxiety and neurodegenerative disease. Physical correlates of changes in brain chemistry, such as changes in cortical representation, have been measured previously in human subjects and animal models but many of the molecular mediators of these changes have yet to be identified (Apkarian *et al.*, 2004b, Apkarian *et al.*, 2004a, Apkarian *et al.*, 2005, Grachev *et al.*, 2000). It has been suggested that chronic pain could be described in terms of memory, where the pain felt is a “memory” of the initial response to tissue injury which for some reason has become more permanent and is maintained by substantial reorganisation within the CNS (Apkarian *et al.*, 2008). Inflammatory mediators have been associated with learning and memory and as such may contribute to the development of this memory and some of the associated physical and psychological symptoms (Ross *et al.*, 2003, Wang *et al.*, 2000, Zeise *et al.*, 1992).

### **9.1.1 Questionnaire studies**

In chapter 4 data was analysed from questionnaires investigating some of the more unusual symptoms seen in CRPS, although the cause of this condition is unknown, dysregulation within the CNS has been implicated (Birklein, 2005). Although the design of the study had some flaws it is still possible to conclude that some degree of cortical reorganisation occurs in CRPS due to the prevalence of reported referred sensations and related symptoms.

These results also emphasised many of the significant “non-painful” symptoms in CRPS which are often overlooked both in this and many other conditions. More research into these symptoms, which have many similarities to conditions involving neurodegeneration and inflammation within the CNS, is vital to provide better insight into the role of the CNS in chronic pain. Such research may open up new avenues in treatment strategies for chronic pain patients focussing, not only on the “sensory-discriminative” elements of the condition, but also on the “motivational-affective” symptoms.

The results of this work prompted the design of an animal model to investigate the effect of persistent inflammatory pain on the brain.

### **9.1.2 Development of an animal model**

Two inflammatory models were investigated in Chapter 5 using CAR and CFA to induce unilateral footpad inflammation in order to develop a pain model that would persist for at least two weeks. CAR and three concentrations of CFA were compared. Consistent with previously published data CAR swelling peaked at three hours post-injection and rapidly returned to baseline, this swelling has been demonstrated to correlate with the development and cessation of hyperalgesia and allodynia and as such this was not considered a suitable pain model for this study (Morris, 2003, Iadarola *et al.*, 1988) since these parameters return to baseline by 7 days post-injection (see figure 14).



CFA injection produced a more persistent and significant increase in paw volume and the 0.1% concentration was chosen as this produced significantly different results to CAR, 0.1% and 1%CFA and persisted for 7 days with no apparent systemic involvement (see figures 13, 15, 16 and 19). Also previous work has demonstrated that at this concentration swelling correlates with hyperalgesia and allodynia with all parameters measures showing an initial peak at 6-24 hours post-injection followed by a decrease to 72 hours and a gradual increase to 14 days and beyond (Stein *et al.*, 1988). It is hypothesised that the initial peak is the result of an acute inflammatory response to a foreign substance, as seen in the CAR model, which rapidly subsides resulting in a minor decrease in paw volume at 72 hours. From 72 hours post-injection swelling increases up until at least 7 days as a result of the cell mediated immune response which has been shown to continue for up to one month (Stein *et al.*, 1988, Raghavendra *et al.*, 2004, Nagakura *et al.*, 2003). This model was then used to investigate the gene expression of a variety of inflammatory mediators using RT-PCR.

### **9.1.3 Cytokine mRNA Expression**

The cytokines IL1 $\beta$  and TNF $\alpha$  were chosen because they have previously been demonstrated to play a role in mediating pain responses in the spinal cord as well as being up regulated in certain brain regions in both neuropathic and inflammatory pain models, they are also known modulators of both neuronal and glial function (Ross *et al.*, 2003, Wang *et al.*, 2000, Ignatowski *et al.*, 1999, Covey *et al.*, 2000). The profile of IL1 $\beta$  expression was not as expected with no increase seen until 7 and 14 days post-injection, in fact, there was a drop in expression in the posterior regions of the brain at 6 -24 hours post-injection (see figures 27 and 28). Interestingly, a similar pattern was seen with TNF $\alpha$  expression (see figures 30 and 31), TNF $\alpha$  is known to mediate IL1 $\beta$  expression so one potential hypothesis is that the drop in TNF $\alpha$  is limiting excessive IL1 $\beta$  release. However, there was one significant anomaly in the expression profile of TNF $\alpha$  mRNA; a large increase was seen 72 hours post-injection in the right posterior region of the brain which had no apparent effect on IL1 $\beta$  expression. The increase in TNF $\alpha$  is

unlikely to be due to activation of ascending neurons and subsequent glial activation since sensory information from the injected paw is represented on the left side of the brain. Therefore it is proposed that this upregulation of TNF $\alpha$  mRNA is the result of activation in descending pathways which may act to increase or decrease the nociceptive process in the injured paw. The overall decrease in IL1 $\beta$  and TNF $\alpha$  mRNA seen in the early time points may be some form of endogenous analgesia, or an attempt to protect the brain from the increased input from the spinal cord.

The elevated levels of IL1 $\beta$  mRNA seen at 7 and 14 weeks imply that this cytokine plays a role in the modulation of longer term pain. As mentioned above IL1 $\beta$  is known to have a significant effect on learning and memory, it is hypothesised that here the cytokine is contributing to LTP and the development of a pain memory which could ultimately result in chronic pain.

It is also possible that persistent low-grade peripheral inflammation has gradually decreased the integrity of the blood brain barrier over time so that by 7 to 14 days post-injection cytokines circulating within the periphery are entering the CNS.

#### **9.1.4 Chemokine mRNA Expression**

This work provides the first evidence for a change in the expression of the chemokine fractalkine (CX3CL1), its receptor (CX3CR1) and another chemokine, growth related oncogene (CXCL1) in the brain in a model of persistent pain (see figures 32-37). Both have previously been implicated in regulation of pain at the level of the spinal cord and in the periphery and altered expression in the brain in neurodegenerative disease has previously been reported (Baliki *et al.*, 2003, Axen and Porath, 1966, Cunha *et al.*, 2008, Han and Neugebauer, 2005, Milligan *et al.*, 2005). A large increase in CXCL1 mRNA expression was seen at 6 hours post-injection suggesting this chemokine is involved in the acute response to a painful stimulus.

CXCL1 release has been associated with an increase in astrocyte activation in multiple sclerosis (Omari *et al.*, 2005) and an increase in astrocyte activation was indeed seen here at 6 hours, however, although CXCL1 mRNA expression decreased significantly at 24 hours post-injection GFAP staining for astrocyte activity remained high. This may be due to negative feedback mechanisms resulting from a large release of CXCL1, it is also possible that the protein is still being released from activated astrocytes at this time point but that mRNA synthesis has decreased. A third suggestion is that the CXCL1 is released as an acute protective response to the initial insult since, in multiple sclerosis for example, this chemokine has been suggested to have a protective role against neurodegeneration (Omari *et al.*, 2005).

It should be noted, however, that the incredibly rapid increase in CXCL1 mRNA expression described here may not be a direct result of the peripheral inflammation but a response to the mechanical insult of the injection itself. If this were the case then it could not be said this chemokine plays a role in inflammatory pain specifically but that it is involved in the CNS response to a painful insult. This hypothesis could be investigated with the addition of an injected control group.

This is the first evidence of differential expression of CX3CL1 and its receptor CX3CR1 in the brain in a persistent pain model. Furthermore, in previous models, where alterations in Fractalkine regulation have been demonstrated within the spinal cord, there is an increase in both the chemokine and its receptor whereas here a significant decrease was seen in the posterior regions of the brain at 6 hours post-injection with levels remaining low until 7 days post-injection. It may be that the decrease in expression seen here is due to down regulation of the ligand and receptor due to negative feedback as a result of release of fractalkine from stimulated neurons.

### 9.1.5 Hif1 $\alpha$ Gene Expression

No significant differences were seen in expression of Hif1 $\alpha$  mRNA expression (see figure 29). As well as playing a role in inflammation Hif1 $\alpha$  has been implicated in regulating memory formation in healthy mice and as such may contribute to the formation of altered pain pathways (Adamcio *et al.*, 2010). Although no real increase was seen here it is possible that in a pathological situation the increase would be greater. Hif1 $\alpha$  is normally expressed in an unstable form under normoxic conditions where it is constantly degraded by the enzyme HIF prolyl-hydroxylase which uses oxygen as a substrate (Semenza, 2004), under hypoxic conditions Hif1 $\alpha$  is stabilised due to inactivation of HIF prolyl-hydroxylase. It has recently been shown that Hif1 $\alpha$  is also modulated by nuclear factor  $\kappa$ B under normal oxygen pressure and that TNF $\alpha$  can regulate this process (van Uden, Kenneth, Rocha 2008), these findings suggest that Hif1 $\alpha$  may play role in the regulation of pain within the CNS even in the absence of hypoxia. Further investigation of the expression of HIF 1  $\alpha$  and related molecules at the protein level may provide further evidence of a role in regulating pain and inflammation.

### 9.1.6 GFAP Expression

Within both sides of the brain GFAP expression, as measured by immunohistochemistry was upregulated at all time points post-injection other than 72 hours with the highest upregulation seen at 24 hours and 14 days post-injection (see figures 39 and 40 and tables 6 and 7). This expression profile correlates with the profile of paw swelling described in Chapter 5 and reported measurements of hyperalgesia and allodynia in previous work.

The increase in activated GFAP on both sides of the brain is evidence to suggest that it may be representative of an increase in glial cross-talk within the CNS, indeed, Raghavendra *et al* (2004) reported an increase in hyperalgesia in the non-injected/contralateral paw at 14 days post-injections suggesting that these CNS alterations are having a behavioural effect. This

communication and spreading of activity within the inflammatory cells within the CNS is likely to be a key component in the plasticity seen in chronic pain within this region, increasing glial activation has a significant effect on neuronal activity and the release of inflammatory mediators may contributed to LTP.

### **9.1.7 Hypoxyprobe Expression**

Although the results of Hypoxyprobe immunohistochemistry were unexpected, with the highest levels of hypoxia observed in the non-injected controls, they provide evidence towards an interesting hypothesis. If hypoxia is induced in non-injected controls, but not CFA treated animals, as a result of the experimental method something must be reversing this hypoxia in CFA-treated animals. Inflammation can be associated with the release of reactive oxygen species (ROS) which can lead to oxidative damage, a process which has been demonstrated to play a role in neurodegenerative diseases such as Alzheimer's and Parkinson's (Higgins *et al.*, 2010).

One hypothesis is that ROS are released in the brain during the acute and sub-acute stages of this pain model, in response to the increase in inflammatory mediators from activated astrocytes and potentially microglia, reversing the hypoxia that is seen in non-injected animals. At later stages hypoxia may be seen due to swelling or tissue damage within the brain as a result of the longer term inflammatory insult. The release of ROS may also contribute to the early stages inflammation and provides a potential mechanism for the neurodegeneration described by previous authors (Apkarian and Scholz, 2006, Apkarian *et al.*, 2004b).

### **9.1.8 Conclusions**

The results presented here provide some interesting insights into changes in the brain seen as a result of peripheral inflammatory pain and certainly warrant further investigation. The first evidence for differential regulation of the chemokines CXCL1 and CX3CL1 is presented providing a potential

mechanism for neuronal-glial communication with the brain. A role for differential regulation of oxygenation is also discussed due to changes seen in expression of a cellular hypoxia marker, Hypoxyprobe.

Although these and related results provide an insight into the process of pain regulation it is important to note that any processes observed here and in similar publications are models of the pain response in normal animals. Although they are excellent models to identify potential biomarkers of pain, it is likely that, in the chronic condition, the processes described here are dysregulated. For example, perhaps in chronic pain CXCL1, seen here to be up regulated in the acute phase, remains elevated leading to a persistent response even after healing. For this reason it is important to endeavour to link animal and cell based studies with clinical trials wherever possible.

## **9.2 Further Work**

To further expand this work it would be interesting to compare and inflammatory model (such as asthma/allergy) with an inflammatory pain model to ascertain if the effects seen are due to the pain itself or the inflammatory response induced by CFA. Adding a control group of saline injected animals would make it possible to elucidate whether acute changes in brain chemistry, as seen here with CXCL1, are in response to the acute painful stimulus of the injection itself or are due to an early inflammatory response to the inflammogen. Building on this it would also be of benefit to compare a neuropathic pain model with inflammatory pain.

Further immunohistochemical studies, perhaps using perfused wax embedded tissue, would allow for more precise identification of specific alterations within the brain and better comparisons between the left and right hemispheres. In combination with this, real time PCR on specific regions (for example, ACC, Insular cortex, thalamus) isolated by microdissection may help build on the expression profile of inflammatory mediators described here. As well as measuring the cytokines and chemokines discussed in this

work it would also be of interest to measure expression of ROS to find out if they are indeed increased in pain models as postulated here.

Utilising the cell model described in Chapter 8 several studies could be performed to identify some of the mechanistic processes behind the differential release of inflammatory mediators. It would be interesting to investigate the effects of both CXCL1 and CX3CL1 on the release of IL1 $\beta$  and TNF $\alpha$  and HIF1 $\alpha$  expression and of these inflammatory mediators on ROS expression. To investigate the potential role for hypoxia in regulating inflammatory mechanisms within the CNS experiments could be carried out in varying oxygen tensions.

Interventional studies need to be performed in order to elucidate the regulatory mechanisms behind the changes in CNS chemistry described here. Administration of the glial inhibitor minocycline at various time points in the animal model described here would identify which, if any, inflammatory modulators are released as a result of glial activation. A beneficial role for minocycline would be of significant interest in this and related areas of research as it is already approved for use as an antibiotic and has excellent blood brain barrier penetration making it a potential candidate for the treatment of chronic pain.

Finally, when a potential biomarker of a painful condition is described in an animal or *in vitro* model it does not necessarily translate to what is seen in the human condition. Wherever possible such models should be combined with data from human experiments, of significant benefit here would be obtaining profiles of inflammatory mediators from the CSF of chronic pain patients. This would provide evidence of circulating biomarkers within the CNS in order to identify potential drug targets based on the molecules identified through basic science.

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